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IN THE U.S. PATENT AND TRADEMARK OFFICE

In re application of

Jacques BARTHOLEYNS — Conf. 1526

Application No. 10/069,575 — Group 1651

Filed June 12, 2002 — Examiner Ruth A. Davis

HUMANIZED BIOMATERIALS, A PROCESS FOR THEIR PREPARATION AND THEIR APPLICATIONS

DECLARATION

Assistant Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Dr. Jacques BARTHOLEYNS, hereby declare:

I am the same Dr. Jacques Bartholeyns named as an inventor in the above-identified patent application. I make this declaration in support of the present application, and to provide evidence in rebuttal to issues raised in the the Official Action of May 12, 2004, that one of ordinary skill in the art would find that NAUGHTON et al. or LEE et al. anticipate or render obvious the claimed invention.

The role of macrophages in tissue repair is believed to be linked to their inflammatory activity, i.e., releasing factors attracting other proliferating cells rather than the macrophages exhibiting a direct action. The fixation of *ex vivo* differentiated, non-proliferating macrophages to a biomaterial therefore induces indirect effects favoring the proliferation of tissue cells. This is clearly different from NAUGHTON et al. or LEE et al. who are colonizing biomaterials with proliferating

cells eventually contaminated with macrophages corresponding to the tissue targeted.

The macrophages from the present invention are derived under *ex vivo* and non-adherent conditions from blood monocytes. In particular, the macrophages are obtained by *ex vivo* differentiation from blood monocytes that leads to living macrophages, and are cultured under conditions enabling their penetration and adherence into biomaterial, for several hours at 37°C, with the porous biomaterial, allowing infiltration of the biomaterial and a substantially irreversible binding of the living macrophages to the biomaterial. The macrophages are humanized with patient's macrophages and ready for implantation.

Thus, the macrophages of the present invention have not encountered tissue specificities before and therefore present different phenotypic and functional characteristics from tissue resident macrophages present in low numbers in the colonizing cells previously described. They can colonize biomaterials implanted thereafter in any tissue and evolve towards macrophages with the implanted tissue specificity.

In addition, important characteristics of the human blood monocyte derived macrophages of the present invention are that these cells have been cultivated in suspension without any adherence step, in contrast to tissue macrophages and to monocytes classically isolated by adherence on membranes. Therefore, our macrophages have kept all adhesins unbound and when penetrating the biomaterial, the macrophages firmly and irreversibly adhere to and colonize the porous surface. These macrophages do not proliferate but will survive for a very long time (we have documented at six months) after binding.

The macrophage suspensions described here release

angiogenic and chemotactic factors which will attract relevant endothelial, muscular or connective tissue cells which will surround the biomaterial and favor a long functionality. This is distinct from NAUGHTON et al. and LEE et al., where the cells derived from a specific tissue used to colonize the three-dimensional scaffold are themselves supposed to directly fulfill a tissue specific reparation role and not a role in the humanization of the material allowing further synergy after implantation.

Indeed, recent publications cited below confirm that properly differentiated macrophages do support tissue repair locally, and that the ex vivo differentiation process can result in TH2 type macrophages distinct from classical macrophages as follows:

Myelomonocytic cells are sufficient for therapeutic cell fusion in the liver...Willenbring H/Bailey AS/Foster M/Akkari Y/Dorrell C/Olson S/Finegold M/Fleming WH/Grompe M., *Nat. Med.*, 10:744-748, 2004.

In Summary, tissue repair (in the present case liver repopulation) occurs after injection of stem cells by fusion between these hematopoietic cells and tissue cells (hepatocytes). This study shows that macrophages derived from the progenitors can produce functional epithelial cells by *in vivo* fusion. Myeloid cells and more particularly macrophages are proposed as a cell therapy strategy to repair damaged organs.

Features of skin-coincubated macrphages that promote recovery from spinal cord injury..., Bornstein Y/Marder JB/Vitner K/Smirno I/Lisaey G/Butovsky O/Fulga V/Yoles E., *J. Neuroimmunol.*, 142: 10-16, 2003.

Purified monocytes were co-incubated with small pieces

of skin for 16 hours. These monocytes acquired some antigen presenting cell markers and secreted cytokines different from the activated macrophages secretions. Infusion of these monocytes (0.2 million) in rats lesion after spinal injury reduced the cavity in the lesion and improved locomotion activity. The authors propose that controlled inflammation can improve tissue repair.

Alternative activation of macrophages...Gordon S., *Nat. Rev. Immunol.*, 3: 23-35, 2003.

TH1 cells induce macrophages cytotoxic activation in an IFN gamma dependent pathway. Alternative macrophage activation by TH2 cytokines does not lead to cytotoxic macrophages, but rather to macrophages participating to tissue repair. Tumor associated macrophages have no killing activity and are TH2 polarized.

The undersigned declares that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

12/10/04
Date

Dr. Jacques BARTHOLEYNS

ALTERNATIVE ACTIVATION OF MACROPHAGES

Siamon Gordon

The classical pathway of interferon-γ-dependent activation of macrophages by T helper 1 (T_H1)-type responses is a well-established feature of cellular immunity to infection with intracellular pathogens, such as *Mycobacterium tuberculosis* and HIV. The concept of an alternative pathway of macrophage activation by the T_H2-type cytokines interleukin-4 (IL-4) and IL-13 has gained credence in the past decade, to account for a distinctive macrophage phenotype that is consistent with a different role in humoral immunity and repair. In this review, I assess the evidence in favour of alternative macrophage activation in the light of macrophage heterogeneity, and define its limits and relevance to a range of immune and inflammatory conditions.

Our understanding of 'classical' immune activation of macrophages dates from studies in the 1960s by Mackaness and colleagues[1], who showed that infection of mice with *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) or *Listeria monocytogenes* enhanced the antimicrobial activities of macrophages in a stimulus-dependent, but antigen-non-specific, manner[2–4]. Studies since then have shown that such macrophage activation depends on the products of specifically activated T helper 1 (T_H1)-type lymphocytes and natural killer (NK) cells — in particular, interferon-γ (IFN-γ)[5] — and a cytokine network involving interleukin-12 (IL-12) and IL-18, which are produced by antigen-presenting cells (APCs). Subsequent studies with knockout mice and in humans with genetic deficiencies of these cytokines and their receptors have validated the importance of this pathway in cellular immunity, immunodeficiency syndromes including AIDS, delayed-type hypersensitivity responses and tissue damage, as in rheumatoid arthritis.

Less well defined is the alternative activation of macrophages by IL-4 and IL-13, cytokines that are produced generally in T_H2-type responses, particularly in allergic, cellular and humoral responses to parasitic and extracellular pathogens. Most early studies grouped the effects of these cytokines on macrophages, together with the effects of IL-10, as being deactivating. However, it has been evident for some time that IL-4 and IL-13 induce overlapping cell-surface and other phenotypic changes that are distinct from those induced by IFN-γ (classical activation) and IL-10 (true deactivation of the respiratory burst and of inflammatory cytokine production, particularly tumour-necrosis factor (TNF), by macrophages). IL-4 and IL-13 upregulate expression of the mannose receptor and MHC class II molecules by macrophages, which stimulates endocytosis and antigen presentation, and they induce the expression of selective chemokines — macrophage-derived chemokine (MDC; also known as CCL22) and thymus and activation-regulated chemokine (TARC; CCL17) — and intracellular enzymes, such as arginase, that are implicated in cell recruitment and granuloma formation.

The concept of alternative activation has not been analysed critically in relation to macrophage heterogeneity and differentiation. It provides a predictive model for transcriptome and proteomic studies, and has been less well studied so far than macrophage activation by IFN-γ[6] or direct activation of macrophage gene expression by lipopolysaccharide (LPS) or microbes[7,8]. Studies with the latter (innate activation) stimuli led to the discovery of Toll-like receptors (TLRs) and signalling pathways that are distinct from those used by the IL-4/IL-13 receptor. The availability of single and double IL-4- or IL-13-knockout and IL-4/IL-13 common receptor α-chain (IL-4Rα)-knockout mice has been exploited recently in various parasitic and other infectious disease models. The importance of alternative activation of macrophages

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for host resistance can now be documented in myeloid-cell-specific knockout animals.

In this review, I deal with the background to this topic, relating it to T-cell and macrophage heterogeneity. The role of APCs as initiators of immunity, as well as effector cells, is explored, as is the regulation of alternative activation *in vivo* and *in vitro*. The possible relevance of alternative activation of macrophages to allergic conditions such as asthma, and to tolerance of the histoincompatible fetus, repair, atherosclerosis and tumour immunity is considered.

As with many other models, over-simplification of the concept of alternative activation of macrophages has begun to erode its value. It has been broadened to include a wider range of immunomodulatory stimuli and macrophage responses[9–11], thereby obscuring relevant differences. An important goal of this review is to define the limits of the concept and to clarify issues that require further examination.

Activation and macrophage heterogeneity

Macrophage-family cells (cells of the mononuclear phagocyte system) have marked phenotypic heterogeneity, as a result of cellular differentiation, widespread tissue distribution and responsiveness to many endogenous and exogenous stimuli. A simplified life history of the macrophage and closely related cells is given in BOX 1.

Apart from constitutive and induced migration, the impact on macrophage differentiation of interactions with altered host cells, modified molecules and exogenous agents should be considered. These ligands are recognized by a diverse range of plasma-membrane receptors, resulting in phagocytosis or endocytosis, intracellular signalling and complex changes in gene activation and repression. Induced cell functions include altered adhesion and migration, secretion of various products, and antigen processing and presentation, as well as the activation of effector functions. FIGURE 1 illustrates the type of macrophage activation that is induced by various endogenous and exogenous stimuli. Ligands on altered host cells (apoptotic or necrotic) are recognized by a range of scavenger-type receptors[12], which suppress or induce macrophage inflammatory responses, depending on incompletely understood mechanisms[13]. Exogenous ligands are recognized by a range of receptors that use opsonins, such as antibody[14], complement[15], collectins[16] and LPS-binding protein[17], or by direct recognition of carbohydrates, proteins, lipids and nucleic acids. Our definition of these receptors has improved considerably, providing knowledge of a range of TLRs[18] and lectins[19], and yielding insights into their impact on signalling and the activation of nuclear factor-κB (NF-κB). Less well appreciated is the fact that many of these receptors interact not only with conserved microbial structures, but also with endogenous host molecules, including modified lipoproteins, lysosomal hydrolases, heat-shock proteins and proteinase complexes. These pattern-recognition receptors contribute to clearance from the extracellular spaces and tissue homeostasis, as part of a wider form of host defence.

Macrophage interactions with specific phagocytic or endocytic ligands are influenced markedly by cytokines and non-phagocytic adhesive interactions with the extracellular matrix, which promote complex changes in patterns of macrophage gene transcription[20], as well as post-translational changes in protein expression. The net outcome can be a full-blown activation phenotype, resulting from preceding or co-incident priming by different cytokine stimuli followed by phagocytic challenge, or, conversely, the downregulation of expression of macrophage activation markers, such as the respiratory burst or pro-inflammatory cytokine production. Similarly, complex interactions with other plasma-membrane molecules, such as C1q-receptor-related protein[21], CD47 (REF. 22) or inhibitory receptors[23], can potentiate uptake through phagocytic receptors or can deactivate macrophage effector mechanisms.

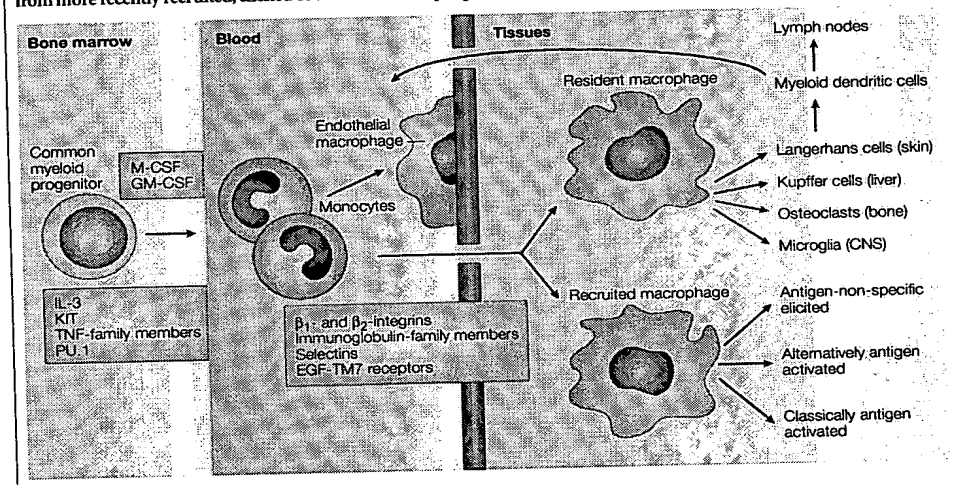
Given the above considerations, it is useful to distinguish the innate (antigen-non-specific, T-cell-independent) immune activation of macrophages from acquired activation, and to incorporate downregulation of macrophage activation in the overall spectrum of responses. Therefore, the interactions of macrophages with activated T and B cells and their products should be seen in a broader context. Immunoglobulins and immune complexes can bind to both activating and inhibitory receptors for Fc[14] and for complement[15], as illustrated by experiments in different mouse-knockout models. Also, Fc-receptor ligation induces marked effects on the release of cytokines, such as IL-12/IL-10 and IL-4, by APCs themselves and by other cells of the innate and acquired immune systems, thereby influencing the subsequent responses of macrophages to antigens or infectious stimuli[24–26].

T-cell heterogeneity in macrophage activation. It is important to distinguish between IFN-γ and IL-4/IL-13 as pleiotropic modulators of macrophage activation that induce distinctive programmes of altered macrophage gene expression after the engagement of their specific cytokine receptors[27,28]. Although it is superficially similar to a T_H2-type cytokine and is often co-induced with T_H2 cytokines in the course of an immune response, it is not appropriate to classify IL-10 together with IL-4 and IL-13 as an alternative activator of macrophages. IL-10 acts on a distinct plasma-membrane receptor to those for IL-4 and IL-13 (REF. 29), and its effects on macrophage gene expression are different, involving a more profound inhibition of a range of antigen-presenting and effector functions, together with the activation of selected genes or functions. Following this line of reasoning, it seems even less appropriate to include the transforming growth factor (TGF) family of cytokines[30], glucocorticosteroids (which are broadlyanti-inflammatory), macrophage colony-stimulating factor (M-CSF), IFN-α/β and TNF in the same category, all of which induce distinct, as well as partially overlapping, patterns of gene expression. Grouping all modulators of macrophages other than IFN-γ into a common alternative-activation group obscures important differences in macrophage responses.

Box 1 | Differentiation, distribution and activation of macrophages *in vivo*

The growth and differentiation of macrophages depends on lineage-determining cytokines, such as macrophage colony-stimulating factor (M-CSF) and granulocyte–macrophage colony-stimulating factor (GM-CSF), and interactions with stroma in haematopoietic organs. Interleukin-3 (IL-3), KIT, tumour-necrosis factor (TNF)-family proteins and TNF-receptor-related molecules contribute to macrophage determination, as do key transcription factors such as PU.1 and other ETS-family members. A common progenitor gives rise to tissue macrophages, myeloid dendritic cells (DCs) and osteoclasts, which are distinct, irreversibly differentiated sublineages. Once distributed through the blood stream, monocytes constitutively enter all tissue compartments of the body. Resident macrophage populations in different organs — such as Kupffer cells (liver), alveolar macrophages (lung) and microglia (central nervous system, CNS) — adapt to their local microenvironment. The signals that are responsible for tissue-specific phenotypes of macrophages include surface and secretory products of neighbouring cells, and extracellular matrix. Adhesion molecules control cell migration from the blood, and through endothelia (as well as trapping endothelium-resident macrophages), the interstitium and epithelia. These include integrins (β_1, β_2 and others), immunoglobulin-superfamily molecules (such as CD31), selectins and epidermal growth factor seven-transmembrane spanning (EGF-TM7)-type receptors related to the F4/80 (EMR1) antigen[107]. Cytokines such as transforming growth factor-β, chemokines and growth factors, often bound to local proteoglycans, influence the expression of a range of macrophage genes. Tissue-resident macrophages cease to proliferate, but they have active messenger RNA and protein synthesis. Although macrophages might die *in situ*, they can be induced to migrate to draining lymph nodes, where they are filtered from the afferent lymph. They do not enter efferent lymphatics or the thoracic duct, unlike DCs. It is possible that monocyte-derived tissue macrophages can re-enter the blood stream and differentiate into DCs, depending on local stimuli such as phagocytosis, leukotrienes and a multidrug-transporter ATPase[108].

Tissue-resident macrophages undergo local activation in response to various inflammatory and immune stimuli; the enhanced recruitment of monocytes and precursors from bone-marrow pools results in the accumulation of tissue macrophages that have enhanced turnover and an altered phenotype. These macrophages are classified as being 'elicited', as in the antigen-non-specific response to a foreign body or sterile inflammatory agent, or as being 'classically activated' or 'alternatively activated' by an antigen-specific immune response. It is difficult to distinguish originally resident macrophages from more recently recruited, elicited or activated macrophages, because cells adapt to a particular microenvironment.



T cells themselves are more heterogeneous than was thought originally[31,32], including not only T_H0-, T_H1- and T_H2-type cells, but also regulatory and possibly T_H3-type cells, some of which secrete TGF-β and IL-10. Individual T cells can produce many cytokines at different stages of differentiation, and T-cell populations *in vivo* are often mixed. Contact-dependent interactions between T cells and macrophages are poorly defined still, but they are known to modulate macrophage activity directly, as well as through cytokines[33,34]. Given these considerations and the fundamental distinction in terms of antigen recognition between T and B cells (which have somatic-recombinant, antigen-specific receptors) and APCs (which have germ-line-encoded, non-clonal receptors that can be modulated markedly in cell populations), I would argue also against the classification of macrophages as M1- or M2-type cells. These cells might be able to change from one phenotype to another, and there is no straightforward correspondence of phenotypes between T-cell subsets and subpopulations of other immune cells. To avoid confusion and mistaken assumptions as to relationships and functions, I therefore propose to limit the term 'alternative activation of macrophages' to the effects of IL-4 and IL-13

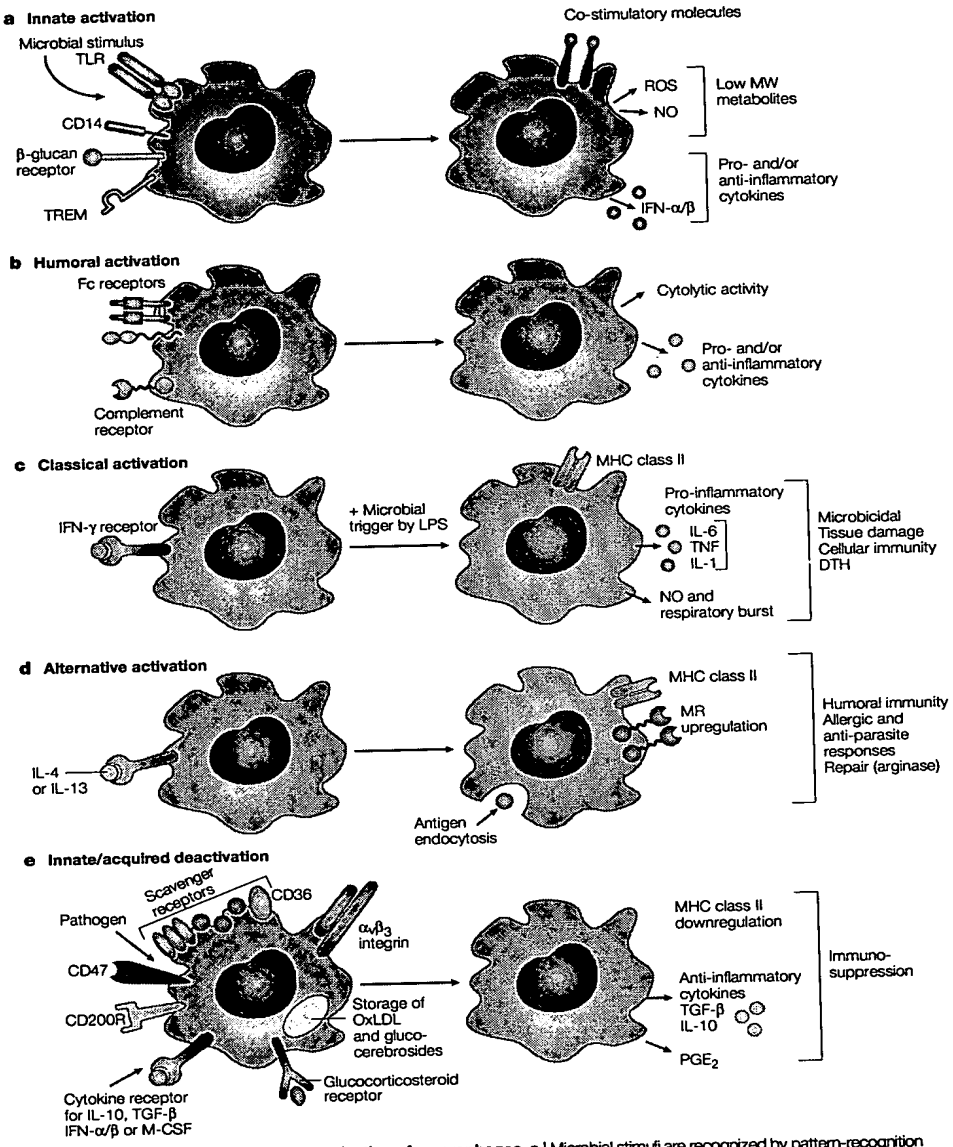


Figure 1 | **Innate and acquired immune activation of macrophages. a** | Microbial stimuli are recognized by pattern-recognition receptors, such as Toll-like receptors (TLRs), CD14/lipopolysaccharide (LPS)-binding protein and a range of non-opsonic receptors. These stimuli induce the production of pro-inflammatory cytokines, such as interferon-α/β (IFN-α/β), and reactive oxygen species (ROS) and nitric oxide (NO), followed by a regulated anti-inflammatory response. Enhanced expression of co-stimulatory surface molecules favours antigen presentation. Scavenger receptor-A (SR-A) and mannose receptor (MR) promote the phagocytosis and endocytosis of host, as well as exogenous, ligands. **b** | Humoral activation and phagocytosis are mediated by some Fc and complement receptors, whereas other receptors downregulate responses. **c** | Classical activation is mediated by the priming stimulus IFN-γ, followed by a microbial trigger (lipopolysaccharide, LPS). **d** | Alternative activation is mediated by interleukin-4 (IL-4) and IL-13, acting through a common receptor chain (IL-4Rα). **e** | Deactivation can be innate or acquired in origin. The uptake of apoptotic cells or lysosomal storage of host molecules generates anti-inflammatory responses. Cellular activity is modulated by the interactions of macrophages with T cells, fibroblasts and matrix, through a range of receptors. Cytokines and glucocorticosteroids are potent modulators of activation. Pathogens can deactivate macrophages by various mechanisms. DTH, delayed-type hypersensitivity; M-CSF, macrophage colony-stimulating factor; MW, molecular weight; OxLDL, oxidized low-density lipoprotein; PGE_2, prostaglandin E_2; TGF-β, transforming growth factor-β; TNF, tumour-necrosis factor; TREM, triggering receptor expressed on myeloid cells.

CRE–LOX TECHNOLOGY
Cre is a site-specific recombinase that recognizes and binds specific sites known as *loxP*. Two *loxP* sites recombine in the presence of Cre, allowing DNA that is cloned between two such sites to be removed by Cre-mediated recombination.

only. Otherwise, it would be necessary to subdivide this category further according to each immunomodulatory cytokine and inhibitor, making the concept unwieldy.

Dendritic-cell heterogeneity. There is now considerable confusion in our understanding of dendritic-cell (DC) heterogeneity and its relationship to the heterogeneity of other APC and T-cell subpopulations. The hallmark of DC function is the unique ability of these cells to present antigens to naive T cells, whereas macrophages and B cells can only activate primed T cells[35]. Recent studies have shown that DCs are functionally heterogeneous, depending on their maturation, interactions with endogenous and microbial stimuli, cell–cell interactions (such as through expression of CD40/CD40 ligand and other co-stimulatory molecules) and cytokine environment (particularly IL-12 and IL-10)[36]. Although DCs are widely accepted as having a crucial role in T_H1-cell versus T_H2-cell differentiation, DC plasticity *in vivo* and *in vitro* makes it inappropriate to equate different types of DC (pre-DC, DC1, DC2 and plasmacytoid DC) with T_H-cell subsets or with corresponding macrophage phenotypes. Depending on their maturity and modulation, DCs can activate or suppress T_H-cell responses, or induce T_H-cell anergy. Also, activated macrophages might share some of these properties, such as the production of immunostimulatory or suppressive molecules and the enhanced uptake of antigens for processing and presentation. At present, it seems preferable to characterize the phenotype and functions of different APCs further, rather than to blur the distinctions between DCs and macrophages.

IL-4 and IL-13 induce alternative activation

It is well established that IL-4 and IL-13 are associated with T_H2-type responses, which have well-defined effects on macrophages, other cells and immune functions (BOX 2). These are outlined here, and the effects of the new T_H2-cell-inducing cytokine IL-25 are also discussed briefly. IL-10, another T_H2-type cytokine, has actions on macrophages that are clearly distinct from those of IL-4 and IL-13 (BOX 3).

The main sources of IL-4 and IL-13 in the body are not well defined, and neither are the stimuli that induce the synthesis and release of these two cytokines. They have broadly similar effects on macrophages and other target cells because they share a common receptor chain, but there are also differences with regard to the range of cell types that respond to each cytokine, which correlates with the presence of different receptor subunits. The intracellular signalling pathway involves members of the Janus-activated kinase (JAK) and signal transducer and activator of transcription (STAT) families[37,38]. Knockout mice are now available for each of these cytokines[39,40] (showing that there is redundancy between them, as well as unique functions for IL-13), for both cytokines[41] and for IL-4Rα[42]. These animals provide well-defined models to study cytokine functions *in vivo*, for comparison with wild-type and IFN-γ- or IFN-γ-receptor-deficient animals, in a context in which T_H-type responses are skewed after an antigenic or infectious stimulus. More recently, IL-4Rα has been ablated selectively in myeloid cells using CRE–LOX TECHNOLOGY, as discussed later.

Effects of IL-4 and IL-13 on macrophages in vitro. The effects of recombinant IL-4 on mouse peritoneal macrophages elicited by biogel-polyacrylamide beads (a sterile, non-immune stimulus) were reported by Stein and colleagues in 1992 (REF. 43). These include a weak proliferative effect (possibly mediated by autocrine M-CSF), the fusion of cells (noted earlier in human macrophages)[44], induction of expression of MHC class II molecules and enhanced levels of macrophage mannose-receptor activity. Although IFN-γ induces the expression of MHC class II molecules, and enhances the macrophage respiratory burst and release of TNF, it selectively downregulates activity of the mannose receptor[45]. Inhibition by IL-4 of the expression of LPS-induced pro-inflammatory cytokines (such as TNF) and of the respiratory burst was moderate, unlike the potent inhibition of these activities, and of MHC class II expression, by IL-10. This pattern of selective activation or inhibition of macrophage gene expression was highly reproducible. When IL-13 became available for experimental use subsequently, the mouse macrophage phenotype induced by this cytokine was shown to be virtually identical to that induced by IL-4 (REFS 46,47). Inhibition of LPS-induced nitric oxide synthase 2 (NOS2) by IL-13 was also moderate, compared with marked inhibition by IL-10, and priming by IFN-γ.

Studies with human blood monocyte-derived macrophages (MDMs) gave broadly similar results, although the production of nitric oxide could not be detected under standard conditions[48,49]. Primary MDMs constitutively express higher basal levels of MHC class II molecules and mannose receptor than do mouse macrophages, and the further upregulation of these markers by IL-4 was less marked. Quantitative studies with mannosylated and other tracers indicated that endocytosis through the mannose receptor was enhanced by IL-4 and IL-13 and that endocytic compartments showed an increased flow of ligands into lysosomes, changes that were distinct from the effects of IFN-γ and IL-10 (REF. 50).

In vitro studies with monocyte-derived DCs have shown that processing and presentation of model antigens can be enhanced by targeted uptake through the mannose receptor[51,52]; however, this has not been shown *in vivo* (DCs *in vivo* do not seem to express mannose receptor constitutively). The role of the mannose receptor in innate and acquired immunity might be considerably more complex than was thought originally[53], as summarized in BOX 4.

Additional markers with a similar pattern of regulated expression (induction by IL-4 and IL-13, with or without downregulation by IFN-γ) have been documented (TABLE 1). These include chemokines, soluble cytokine receptors and other proteins. The first transcriptome studies have been reported recently[54], but no proteomic analysis has been carried out so far.

Apart from possible roles in antigen presentation and APC migration, the effects of IL-4 and IL-13 on

CHROMATIN IMMUNOPRECIPITATION (ChIP). The use of antibodies specific for transcription factors to precipitate nucleic-acid sequences from chromatin for amplification.

arginine metabolism and macrophage effector functions are particularly marked. Both cytokines stimulate arginase activity *in vitro*, thereby counteracting the effects of NOS2 activation and nitric oxide release; this might be relevant to the fibrosis and repair of granulomatous inflammation *in vivo*, as discussed later.

Recent studies have begun to explore the effects of cytokines on T-cell and macrophage chromatin structure, histone acetylation and access to transcription factors[55–57]. The alternative activation of macrophages by IL-4 and IL-13 provides an excellent model system for CHROMATIN IMMUNOPRECIPITATION (ChIP) studies under defined conditions *in vitro* and for the analysis of gene activation.

Effects of IL-4/IL-13 on IL-1 and chemokines. IL-4 and IL-13 participate in $CD4^+$ T-cell priming, memory and effector functions, and in the T_H1–T_H2 balance and selective recruitment, directly and through APCs[58]. Mantovani's group has made important contributions to this topic, studying mainly human mononuclear cells *in vitro*. IL-4 and IL-13 augment expression of IL-1 decoy receptor and the IL-1 receptor α-chain *in vitro* and *in vivo*, thereby counteracting the pro-inflammatory actions of IL-1 (REFS 59,60). They also upregulate the expression and release of CCL22 (inhibited by IFN-γ and IL-10), which attracts CC-chemokine receptor 4 $(CCR4)^+$ T_H cells, thereby amplifying polarized T_H2 responses[61,62]. CCL17 is similarly upregulated by IL-4 in monocyte or DC culture models, and it acts on $CCR4^+$

Box 2 | Comparison of IL-4 and IL-13

Biochemical features
- Proteins: interleukin-13 (IL-13) shares several structural characteristics with IL-4 and belongs to the same a-helix protein family.
- Genes: the genes encoding IL-4 and IL-13 have ~30% sequence homology and a related intron/exon structure, and they are adjacent in a syntenic region on human chromosome 5 and mouse chromosome 11. A coordinate regulator of the expression of IL-4, IL-13 and IL-5 has been discovered by cross-species sequence comparisons[109] and by genetic deletion[110].

Sources
- Various sources, including T helper 2 (T_H2) cells (IL-4), T_H0, T_H1 and T_H2 cells (IL-13), mast cells and basophils.
- The expression of IL-4 is regulated by distal elements located in the *IL-4* locus, through which GATA3 acts at the chromatin level[111].
- Profound increases in histone acetylation occur at the *IL-4* locus during T_H1/T_H2-cell differentiation[112].
- The production of IL-4, but not IL-13, depends on calcineurin/calmodulin pathways.

Target cells
- Monocytes and B cells — IL-13 has IL-4-like effects.
- T-cell differentiation is induced by IL-4, but not by IL-13.
- IL-4 induces characteristic T_H2 responses even in the combined absence of IL-5, IL-9 and IL-13 (REF. 113).

Receptors
- IL-13 binds to three receptor complexes — the low-affinity IL-13 receptor-α1 (IL-13Rα1) monomer, the high-affinity IL-13Rα–IL-4Rα heterodimer and the IL-13Rα2 monomer (the genes encoding IL-13Rα1 and IL-13Rα2 are closely linked on the X chromosome).
- IL-4 binds to two receptor complexes — the high-affinity heterodimer of IL-4Rα and the IL-2R common γ-chain, which is expressed to different degrees by T and B cells, mast cells and macrophages, and the IL-4Rα–IL-13Rα1 heterodimer, which is expressed by B cells, monocytes, endothelia, epithelia and fibroblasts.

Signalling
- IL-4Rα is a signal-transducing receptor for IL-4 and IL-13, through signal transducer and activator of transcription 6 (STAT6) or insulin receptor substrate 2 (IRS2) pathways.

Effects and functions
- Overlapping, cooperative, non-redundant functions in T_H2-cell polarization, disease susceptibility and protection against selected parasitic infections.
- Both cytokines antagonize the actions of IFN-γ.
- Both cytokines can act with granulocyte–macrophage colony-stimulating factor to induce the differentiation of immature dendritic cells to cells with enhanced antigen-presenting function.
- Both cytokines can enhance B-cell class switching and synthesis of immunoglobulin E and other antibody subclasses.
- IL-13 has specific effects on mucus secretion and tissue eosinophilia in asthma and parasitic infections.

For further details, see REFS 114,115. Common effects of these cytokines on macrophages are shown in TABLE 1. Other immunostimulatory and immunosuppressive effects on natural killer cells, neutrophils, mast cells, smooth muscle cells, endothelial cells and fibroblasts have been reported.

Box 3 | IL-10 and IL-4/IL-13: distinct effects on macrophages

- Macrophages are an important source, as well as target, of interleukin-10 (IL-10). The functional IL-10 receptor (IL-10R) consists of the ligand-binding subunit IL-10R_1 and the accessory subunit IL-10R_2. Whereas IL-10R_2 is expressed by most cells, IL-10R_1 is expressed by haematopoietic cells and is upregulated by macrophages after activation.
- IL-10 (REFS 29,116) has different morphological effects to IL-4/IL-13 on macrophages (for example, rounding versus fusion); it downregulates the expression of MHC class II molecules and has variable effects on mannose-receptor expression. Fluid-phase and mannose-receptor-mediated endocytosis are reduced by IL-10, compared with their induction by IL-4/IL-13, with marked differences in flow through early and late endocytic compartments[50].
- IL-10 is a potent signal transducer and activator of transcription 3 (STAT3)-dependent inhibitor of pro-inflammatory cytokine production and nitric oxide release, after challenge with lipopolysaccharide (LPS). IL-10-deficient mice develop widespread inflammatory-cell infiltrates, including in the bowel, and transgenic animals that constitutively overexpress IL-10 in macrophages suffer from septic shock and overactivity of pro-inflammatory cytokines[117].
- Gene-array studies show a selective pattern of gene expression in bone-marrow-derived macrophages (from IL-10-deficient mice) treated with exogenous IL-10, with or without LPS[118]. Upregulated target genes include those encoding IL-1Rα, CD32, CC-chemokine receptor 5 (CCR5) and CCR1, as well as suppressor of cytokine signalling 3 (SOCS3). The profile of global gene expression is distinct from that induced by IL-4/IL-13, although no direct gene-array comparison has been reported.
- The upregulation of expression of IL-4Rα by IL-10 correlates with increased IL-4-dependent expression of arginase-1. IL-10 also synergizes with LPS to increase the expression of arginase-2. Therefore, IL-10 increases the total level of arginases in macrophages in many ways[118].

subsets of $CD4^+$ T cells with the potential to produce T_H2 cytokines[63]. These differential effects can be contrasted with those of IFN-γ-induced protein 10 (IP-10; CXCL10), which shows some preference for T_H1 cells, and of IL-10, which can uncouple the function of CCR5 from enhanced expression, consistent with its anti-inflammatory functions. Furthermore, IL-4 and IL-13 enhance the functions of CXCR1 and CXCR2 and render monocytes highly responsive to IL-8 (CXCL8) and related chemokines[64].

Macrophages and pathogenesis of disease

T_H2-cell-mediated infection and allergy. Alveolar macrophages, as resident cells of the lungs, have a distinctive phenotype compared with other resident macrophages in the body, express high levels of pattern-recognition receptors — mannose receptor, scavenger receptor-A (SR-A) and the β-glucan receptor — and constitutively secrete large quantities of lysozyme[65]. They express messenger RNA encoding a range of pro-inflammatory cytokines, perhaps as a result of stimulation by particulates, and they also secrete various immunosuppressive products. They are, therefore, central to innate defences of the airways, with important contributions from epithelial and type II alveolar cells, and they modulate lung reactivity in acquired immunity. Monocytes are recruited readily to the lungs as part of T_H1- and T_H2-type inflammatory responses to pathogens and allergens. Recent studies have established the importance of chemokines — including monocyte-chemotactic protein 1 (MCP1; CCL2), macrophage inflammatory proteins (MIP1α or CCL3; MIP1β or CCL4; and MIP2 or CXCL2) and CXCL10 — and chemokine receptors (CCR1 and CCR2) in various mouse models of infection with *Aspergillus fumigatus* or *Cryptococcus neoformans*, and in *Schistosoma mansoni* egg antigen (SEA)-induced granulomata[66,67]. CCL2 is essential for T_H2 responses, and its overexpression is associated with defects in cell-mediated immunity[68,69]. However, CXCL10, a prototypical T_H1 product, is also implicated in T_H2 inflammation[70], and CCR2, a receptor for CCL2, has an important role in the T_H1–T_H2 balance.

Many studies have shown that asthma and related models involve T_H2-type cell recruitment and cytokines, which influence airway reactivity, mucus production and local connective-tissue turnover. IL-4 and IL-13 have both been implicated; the local overexpression of IL-13 in transgenic mice has marked T_H2-type effects, even without an exogenous stimulus, and many of these effects depend on the involvement of CCR2–CCL2 interactions.

Recently, a new T_H2-type cytokine, IL-25, has been shown to be produced by highly polarized T cells[71]. Although this cytokine is structurally related to IL-17, it has markedly different properties. The infusion of IL-25 into mice induced the expression of IL-4, IL-5 and IL-13, resulting in increased serum levels of immunoglobulin E, IgG and IgA, eosinophilia and pathological changes in lungs and the digestive tract, possibly due to overexpression of local IL-13. Signalling through IL-4Rα is required for some of the effects. The source of IL-25 and its target cells have not been identified, but the latter might be rare accessory cells that have high-level expression of MHC class II molecules, but low-level expression of CD11C and lineage-specific markers. Direct effects of IL-25 on macrophages have not been reported.

Perhaps the best characterized T-cell-dependent model of differential macrophage activation *in vivo* is that of *Mycobacterium bovis* purified protein derivative (PPD) (T_H1) and SEA (T_H2) granuloma formation in mice, investigated by Kunkel and colleagues[72–74]. Sepharose beads coated with PPD or SEA are injected intravenously into presensitized mice. Lung granulomata, which are formed by the immune-mediated recruitment of white blood cells, are particularly rich in macrophages. The granulomata are analysed morphometrically, their cell content is determined and the levels of various cytokines, receptors and biochemical parameters of inflammation and repair are measured in the lungs, as well as draining lymph nodes. The evolution of typical T_H1- and T_H2-type granulomata, the latter having more extensive fibrosis, follows a reproducible time course. Antibody treatments (to block IL-4, IL-13 or both), recombinant cytokines, soluble receptors and *Pseudomonas* exotoxin conjugates have been administered to manipulate granuloma formation. A second T_H2-granuloma model, used extensively by many groups, consists of the intravenous injection of *S. mansoni* eggs, which lodge in the liver as well as the lungs. This model has been studied in various wild-type and knockout mice[75].

Box 4 | Immunological functions of the mannose receptor

- The mannose receptor[53] is a type 1 transmembrane glycoprotein with eight C-type lectin carbohydrate-recognition domains and a cysteine-rich domain[119].
- It is expressed by macrophages, and selected endothelial and dendritic cells, but not by monocytes or neutrophils.
- It mediates the endocytosis and phagocytosis of mannosyl glycoconjugate ligands *in vitro* and *in vivo*[120]. Natural ligands that are cleared by the mannose receptor include lysosomal hydrolases, tissue plasminogen activator, myeloperoxidase and thyroglobulin. Microbial ligands include antigens from *Klebsiella*, yeast and HIV[19].
- Mannose-receptor endocytic activity is induced by interleukin-4 (IL-4) and IL-13, and downregulated by interferon-γ *in vitro*.
- Plasma-membrane mannose receptor is cleaved by a metalloproteinase, and soluble mannose receptor is found constitutively in plasma.
- The cysteine-rich domain of the receptor binds sulphated carbohydrate ligands that are expressed by marginal-zone metallophilic macrophages in the spleen and subcapsular-sinus macrophages in the lymph nodes, as well as the anterior pituitary hormone lutropin[121].
- The mannose receptor might have a role in glycoprotein capture and transport *in vivo*, promoting clearance and reduced immunogenicity (P. Taylor and L. Martinez-Pomares, unpublished observations).

Although IFN-γ (T_H1) and IL-4 and IL-13 (T_H2) have all been implicated in the development of granulomata, a role for chemokines and their receptors — including CCL2, CCR2 and RANTES (regulated on activation, normal T-cell expressed and secreted; CCL5) — in determining the cellular composition of granulomata has been observed also. IL-4 and IL-13 have common, as well as disparate, regulatory functions in type-1 and type-2 responses. These models provide suitable material for further studies into the role and regulation of classically and alternatively activated macrophages *in vivo*, their antimicrobial potential and the cellular interactions that are involved in immune modulation and repair. In addition to chemokines (such as CCL2) and IL-4/IL-13, TGF-β has a key role in fibrosis, acting directly on fibroblasts, as well as through macrophages. By contrast, IL-10 and IFN-γ reduce collagen synthesis.

Alternative activation in parasitic infections

One of the hallmarks of classical activation of macrophages in mice, and probably in humans, is the generation of nitric oxide by an inducible nitric oxide synthase (NOS2), for example in IFN-γ-primed LPS-challenged cells. This is thought to be important for microbial killing, as shown by NOS2-deficient mice, although the NADPH oxidase and oxygen-independent pathways also contribute to this. IL-4 and IL-13 downregulate the synthesis of NOS2 *in vitro* in some macrophage populations; a substrate-competition mechanism, dependent on STAT6, has been put forward also[76]. Studies by Modolell and colleagues[77,78] established that expression of L-arginase is induced in macrophages by T_H2-type cytokines and that the balance between NOS2 and arginase correlates with the balance between T_H1- and T_H2-type activities. Hesse, Wynn and colleagues[79,80] extended this concept to SEA-induced granuloma formation *in vivo*, devising a T_H2-type model and an immune-deviated model incorporating IL-12. Two important conclusions were drawn: first, the anti-inflammatory and anti-fibrotic effects of the type-1 response were markedly dependent on NOS2, as shown by studies of knockout mice; second, IFN-γ and IL-4/IL-13 showed reciprocal inhibition of the activities of arginase and NOS2, respectively. Type-2 cytokine-stimulated macrophages produced proline, an important precursor of collagen, under the strict control of arginase. Other groups have also shown the induction of expression of arginase in models of macrophage activation — the enzyme resembles the hepatic and cytosolic, but not the mitochondrial, forms, and it has been localized immunocytochemically in *S. mansoni*-induced granulomata. Therefore, the alternative metabolic pathways of L-arginine metabolism (FIG. 2) are consistent with the differential activation of macrophages, and they provide a potential explanation for the association of T_H2-type inflammation with enhanced fibrosis.

Brombacher and colleagues have generated macrophage/neutrophil-specific IL-4Rα-deficient ($lysM^{Cre}$, $IL\text{-}4R\alpha^{-1f\alpha\alpha}$) mice, and recently they have analysed two experimental models of disease — infection with *Nippostrongylus brasiliensis* and with *Leishmania major*. Mice with a total deficiency of IL-4Rα could not eradicate *N. brasiliensis* by worm expulsion, and T_H2 responses and goblet-cell hyperplasia were impaired (C. Holscher *et al.*, unpublished observations), whereas selective myeloid deficiency of IL-4Rα had no effect on these activities. By contrast, myeloid-cell responses contributed to host susceptibility to *L. major* in BALB/c mice, and IL-4Rα deficiency was associated with increased T_H1-cell-dependent macrophage effector functions. They concluded that IL-4- or IL-13-stimulated macrophages and/or neutrophils contributed to the susceptibility of the non-healer BALB/c strain to infection with *L. major* not only by reducing classical macrophage activation, but also by suppressing protective T_H1 responses. In the myeloid-targeted knockout mice, macrophages were impaired with respect to IL-4-mediated phosphorylation of STAT6, induction of expression of MHC class II molecules, and suppression of IL-12 p40 production by LPS and/or IFN-γ stimulation, and with respect to IL-13-mediated suppression of nitric oxide production by LPS and/or IFN-γ stimulation. The addition of IL-4 to *Listeria*-infected macrophages from receptor-deficient mice failed to suppress the production of pro-inflammatory cytokines (IL-12 p40, TNF and IL-6, as well as the chemokines CCL3, CXCL2 and CXCL1). Similarly, IL-4 failed to suppress nitric oxide production or parasite elimination of *L. major*-infected macrophages. Control experiments confirmed that lymphocyte responses were unimpaired in these myeloid-specific receptor-deficient mice. This important study distinguishes between the myeloid and lymphoid functions that are mediated by IL-4 and IL-13, and provides a valuable model to define the role of alternatively activated macrophages *in vivo* in a range of infectious and immune pathologies.

An experimental model of mouse trypanosomiasis has also proved to be a useful starting point to explore

Table 1 | **IL-4/IL-13-induced markers of alternative macrophage activation**

Marker*	Comment	References
Receptors		
Mannose receptor	Expression (protein and activity), endocytosis of specific ligand and secretion of soluble receptor increased. Selective downregulation by IFN-γ	43,46,50,53 45
CD23 (FcR for IgE)	Allergy association. IFN-γ upregulates different FcRs	122
CD163	Involved in haptoglobin–haemoglobin clearance. Upregulated by IL-10, glucocorticoids and, perhaps, IL-4	123
Antigens		
MHC class II and some adhesion/costimulatory molecules (CD80/CD86)	Upregulated by IFN-γ	48
MS1-HMWP	Downregulated by IFN-γ. Expressed by selected endothelial cells	124
Adhesion molecules		
Fibronectin splice variant ECM βIG-H3	Expression inhibited by glucocorticoids	125
Chemokines		
AMAC1 (CCL18)	MIP1α like. Also expressed by DCs. Upregulated by IL-10	126
MDC (CCL22)	Downregulated by IFN-γ	61
TARC (CCL17)	Expression controlled by IL-4/IFN-γ in an antagonistic manner by a combined STAT6/STAT1-binding element	63
MCP1 (CCL2)	Complex induction in endothelial cells, but not in monocytes	68,69
Cytokines		
IL-1ra/IL-1 decoy receptor	Anti-IL-1 effects	59
[IL-1, IL-6, TNF]	Counteracts LPS-induced pro-inflammatory effects	114,115
IL-10	Effect varies depending on timing of LPS stimulus	127
TGF-β	IL-13 stimulates and activates TGF-β	128
Metabolic factors		
L-arginase 1	Counteracts NOS2. Expression induced by IFN-γ/LPS	78
12,15-lipoxygenase	Mediates PPARγ upregulation by IL-4. Expression inhibited by IFN-γ	98,129
Products		
FIZZ1	Resistin-like secreted protein. Expression controlled by IL-4/IFN-γ in an antagonistic manner	83
Ym1/2	Closely related soluble chitinase-like lectins. Expression controlled by IL-4/IFN-γ in an antagonistic manner	85
[Collagenase]/MMP1 [MMP9]	Induced by T_H2-cell membrane factors and IL-4 in GM-CSF-differentiated human monocytes	34

*The expression of markers is upregulated except for those given in square brackets. In most cases, both IL-4 and IL-13 have been shown to have similar activity where investigated. I have omitted markers ascribed solely to IL-10 or other macrophage-deactivation stimuli, rather than IL-4. See text for further references. AMAC1, alternative macrophage activation-associated CC-chemokine 1; DC, dendritic cell; ECM, extracellular matrix; FIZZ1, found in inflammatory zone 1; GM-CSF, granulocyte–macrophage colony-stimulating factor; IFN-γ, interferon-γ; IL, interleukin; IL-1ra, IL-1 receptor antagonist; LPS, lipopolysaccharide; MCP1, monocyte chemotactic protein 1; MDC, macrophage-derived chemokine; MIP1α, macrophage inflammatory protein-1α; MMP, matrix metalloproteinase; MS1-HMWP, MS1 high molecular weight protein; NOS2, nitric oxide synthase 2; PPARγ, peroxisome proliferator-activated receptor-γ; STAT, signal transducer and activator of transcription; TARC, thymus and activation-regulated chemokine; TGF-β, transforming growth factor-β; T_H2, T helper 2; TNF, tumour-necrosis factor.

the role of alternatively activated macrophages[81]. Early stages of infection with a *Trypanosoma brucei* variant are characterized by the development of classically activated macrophages, whereas alternatively activated macrophages develop in the late and chronic stages of infection, their appearance correlating with impaired antigen, but not mitogen- or superantigen-, induced T-cell activation. Markers that were used to establish alternative activation included high levels of arginase, low levels of nitrate, and high levels of IL-4, IL-13 and IL-10 in cell culture or plasma. The authors then used SUPPRESSION SUBTRACTIVE HYBRIDIZATION to identify known and new genes that are expressed by different types of activated macrophage[82]. They showed that FIZZ1 (found in inflammatory zone 1; also known as RELMα) and Ym1 are strongly induced in alternatively activated macrophages *in vivo* and *in vitro*, that this induction depends on IL-4 *in vivo* and that IFN-γ antagonizes the effects of IL-4 and IL-13 on the expression of both markers *in vitro*. FIZZ1 is a resistin-like secreted protein, the expression of which is upregulated in alveolar epithelial and type II cells during allergic pulmonary inflammation and is reported to antagonize the effects of nerve growth factor. Ym1 is a chitinase-like secretory lectin that forms crystals in alveolar spaces and in hyperactive lung macrophages and giant cells. These two new markers for

SUPPRESSION SUBTRACTIVE HYBRIDIZATION
A method to isolate unique messenger RNA sequences from paired sources, by subtracting common sequences, for complementary-DNA production.

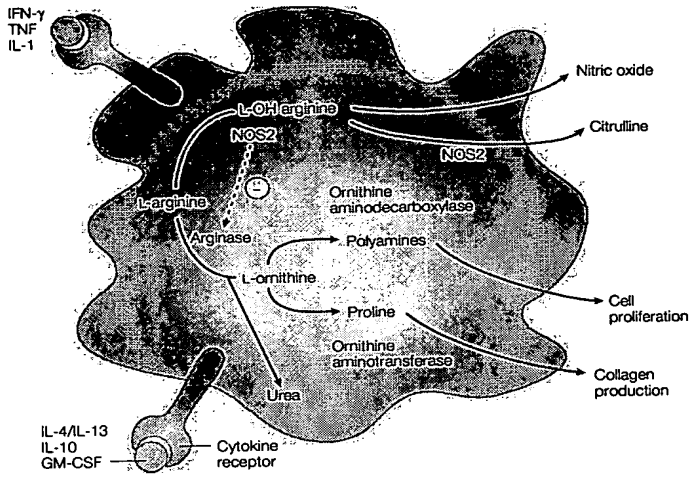


Figure 2 | **Differential utilization of L-arginine by activated macrophages.** Interleukin-4 (IL-4) and IL-13 promote arginase-dependent formation of L-ornithine and, ultimately, fibroblast proliferation and collagen production. Interferon-γ (IFN-γ) enhances the activity of nitric oxide synthase 2 (NOS2) to generate nitric oxide, and inhibits arginase. GM-CSF, granulocyte–macrophage colony-stimulating factor; TNF, tumour-necrosis factor. Adapted, with permission, from REF. 80 © (2001) The American Association of Immunologists, Inc.

alternatively activated macrophages are expressed also by macrophages from mice infected with *Brugia malayi* and *Trichinella spiralis*. Recent microarray studies[83] confirmed that *Ym1* is highly upregulated by IL-4 treatment of mouse peritoneal macrophages, and molecular characterization of the *Ym1* promoter indicated the presence of many STAT6 response elements that are obligatory for cell activation *in vitro* and in an ovalbumin-treated mouse model of allergy. A closely related molecule, Ym2, has been shown to be induced by IL-13 during allergy in the lung[84]. Allen and colleagues[85] have used a comparable nematode infection model to study alternative macrophage activation.

Macrophage activation in other processes

In view of their anti-inflammatory effects and possible role in trophic and repair processes, several authors have proposed that alternatively activated macrophages might contribute to a range of inflammatory and non-inflammatory processes in health and disease. These might include tolerance of the allogeneic fetus, repair (including in the central nervous system, CNS), atherosclerosis and tumour–host-stroma interactions. Generally, these studies lack adequate characterization of the macrophage phenotype *in situ* or after cell isolation, and experimental models have not been established in the knockout mice discussed earlier. Moreover, the definition of alternative activation has lacked precision, being based on the local presence of a broad range of immunomodulatory cytokines, including IL-10 and TGF-β, as well as M-CSF. However, alternative activation of macrophages might contribute to these processes and this hypothesis merits further research.

Fetal tolerance. This remains a mystery, with many hypotheses having been advanced to account for the failure of the mother to reject an allogeneic fetus. Macrophages are prominent in the placenta, amniotic fluid and uterus, and a T_H2 cytokine environment has been postulated in the placenta[86–88]. A recent intriguing theory, backed by experimental evidence, proposes that local macrophages catabolize L-tryptophan, an essential amino acid that is required for T-cell activation, by the upregulation of expression of indole 2,3 dioxygenase (IDO)[89,90]. Administration of an inhibitor of this enzyme resulted in the rejection of allogeneic, but not syngeneic, fetuses. There is no compelling evidence that local macrophages contribute to fetal tolerance by the modulation of T_H2-type cytokines.

Wound repair. Macrophages are important in wound repair; they contribute to fibrin dissolution, the removal of dead tissue and the ingrowth of new blood vessels, and they regulate fibroblast recruitment, growth and connective-tissue remodelling. T-cell-dependent activities are not required, but T cells might have indirect effects on relevant macrophage functions. *In vivo* studies have shown that alternatively activated macrophages are associated with a high degree of vascularization[91]. It remains to be established whether such macrophages can influence wound repair by specific effector mechanisms, as described for granuloma fibrosis[92].

Repair in the CNS. Macrophages can enter the normal CNS, but they fail to respond to pro-inflammatory stimuli such as locally injected chemokines, which is consistent with the existence of a potent, local anti-inflammatory environment in the normal neural parenchyma[93]. This can be overcome by a range of metabolic, infectious and immune stimuli, which result in microglial activation, as well as the enhanced recruitment of monocytes, and which give rise to macrophages with the potential to produce injurious metabolites. Again, evidence that alternatively activated macrophages can counteract cellular or humoral immune injury, and contribute to astrocyte repair processes, is lacking.

Atherosclerosis. This is a chronic disease process in which lipid deposits and superimposed thrombosis in large arteries result in vascular occlusion, giving rise to heart attacks and strokes. Atherosclerosis is now regarded widely as a modified type of inflammation in which recruited monocyte-derived macrophages have a central role through interactions with the endothelium, smooth muscle cells and platelets[94]. The unregulated uptake of oxidized lipoproteins generates foamy, fat-laden macrophages in the arterial wall. Knockout mice that lack apolipoprotein E (ApoE) develop analogous lesions with foam cells in their arteries. Crosses with M-CSF- or chemokine-receptor-deficient animals have provided evidence of a primary role for macrophages in lesion development. IFN-γ potentiates atherosclerosis in ApoE-deficient mice through local effects in the arterial wall, as well as a systemic effect on the levels of plasma

lipoproteins[95]. T_H2-type cytokines and chemokines are present in late stages of the human disease[96]. Experimental evidence is required to support the theory that T_H2 cytokines, perhaps acting through alternatively activated macrophages, can promote plaque stability by favouring fibrous-cap formation, thereby reducing the risk of rupture and local thrombosis. Peroxisome proliferator-activated receptor γ (PPARγ), which is implicated in macrophage inflammatory responses to lipids, has also been linked to IL-4 activity, through enhanced levels of 12,15-lipoxygenase, an enzyme that is involved in arachidonate metabolism[97].

Tumours. Macrophages are present, and are occasionally abundant[98], in a range of tumours, such as breast carcinoma, meningioma and Hodgkin lymphoma[99]. For example, Reed-Sternberg cells, which are characteristic cells of uncertain origin present in Hodgkin lymphomas, have been shown to express CCL22 and CCL17 (REF. 100). These chemokines bind to CCR4, which is expressed preferentially by polarized T_H2 cells and monocytes. Studies of cellular infiltrates indicate that T_H2-type and other cytokines, such as M-CSF and vascular endothelial growth factor (VEGF), and various chemokines that are present locally, are produced constitutively by tumour cells, as well as normal cells. Macrophages are the source of CCL18 in human ovarian ascites[101], and expression of this chemokine by macrophages can be induced by IL-4, IL-13 and IL-10. The differential production of chemokines that attract T_H2 cells or regulatory T cells (such as CCL22) or T_H1-type cells (such as CXCL9 and CXCL10) integrates mononuclear-cell responses in tumours. Tumour-associated macrophages seem to be incapable of killing established tumours, and they might indeed be trophic for nascent tumour clones[102,103]. Interactions with tumour cells, stromal fibroblasts and regulatory T cells could generate an anti-inflammatory T_H2-polarized phenotype of macrophages. Further studies are required to isolate tumour-associated macrophages, to characterize them phenotypically and to establish whether they contribute to tumour progression and metastasis, through the activity of proteolytic enzymes and/or hypoxia-induced angiogenesis[104]. Subsets of 'alternatively activated' macrophages, perhaps representing immature myeloid cells, have been identified in the spleens of lymphoma-bearing mice[105]. These might contribute to NK-cell deactivation in the tumour microenvironment. It will be important to distinguish between IL-4/IL-13, on the one hand, and IL-10/TGF-β/M-CSF/IL-6, on the other hand, as additional modulators of macrophage and DC differentiation and responses in different histological types of tumour.

Therapeutic implications and conclusions

Studies have begun using antibodies, soluble receptors or chimaeric proteins that regulate the function of IL-13 as potential inhibitors to restrict fibrosis in granuloma formation[106]. In situations where alternatively activated macrophages limit tissue injury or promote repair, it might be helpful to augment their activity, for example in stabilizing atherosclerotic plaques. Restricting the definition of alternative macrophage activation to modulation by IL-4 and/or IL-13, as I have done here, should provide a more clearly defined therapeutic target. However, a great deal of experimental work is required still to establish the complex contributions of alternatively activated macrophages to disease pathogenesis. The role of innate recognition receptors of APCs in directing T_H1/T_H2-type acquired immune responses needs to be investigated, and the relationship between IL-4/IL-13 and IL-10 in the phenotypic modulation of macrophages should be clarified, with special reference to contrasting immune enhancing and suppressive actions. There is an urgent requirement for better and additional markers for the *in situ* analysis of alternatively activated macrophages, as well as for FACS (fluorescence-activated cell sorting) analysis of isolated cells. Above all, the complexity of APC phenotypes that are observed *in vivo* needs to be correlated with heterogeneity *in vitro*. Such knowledge is essential for understanding the dynamic changes that macrophage and DC populations show in a range of physiological and pathological situations, as a basis for extensive messenger RNA and protein analysis and for more-selective therapeutic manipulation.

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Myelomonocytic cells are sufficient for therapeutic cell fusion in liver

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Liver repopulation with bone marrow–derived hepatocytes (BMHs) can cure the genetic liver disease fumarylacetoacetate hydrolase (Fah) deficiency[1]. BMHs emerge from fusion between donor bone marrow–derived cells and host hepatocytes[2]. To use such *in vivo* cell fusion efficiently for therapy requires knowing the nature of the hematopoietic cells that fuse with hepatocytes. Here we show that the transplantation into *Fah*$^{-/-}$ mice of hematopoietic stem cells (HSCs) from lymphocyte-deficient *Rag1*$^{-/-}$ mice, lineage-committed granulocyte-macrophage progenitors (GMPs) or bone marrow–derived macrophages (BMMs) results in the robust production of BMHs. These results provide direct evidence that committed myelomonocytic cells such as macrophages can produce functional epithelial cells by *in vivo* fusion. Because stable bone marrow engraftment or HSCs are not required for this process, macrophages or their highly proliferative progenitors provide potential for targeted and well-tolerated cell therapy aimed at organ regeneration.

Hematopoietic stem cells give rise to several nonhematopoietic lineages *in vivo*, including skeletal[3] and cardiac[4] muscle, neurons[5] and epithelial cells such as hepatocytes[1,6–8]. Cell fusion has been identified as the principal mechanism by which HSC-derived hepatocytes[2,9,10], cardiomyocytes[10,11] and Purkinje-cell neurons[10,12] arise. Even the unambiguous finding of cell fusion in HSC-derived epithelial progeny does not, however, formally exclude the possibility of stem cell plasticity: in the liver, HSCs might first differentiate into hepatocytes and subsequently fuse with host hepatocytes.

To determine whether spontaneous hepatocyte fusion occurs in the *Fah*$^{-/-}$ liver repopulation model[13], we carried out serial transplantations of genetically marked hepatocytes (Fig. 1a). We transplanted 1×10^5 wild-type (*Fah*$^{+/+}$) hepatocytes into each of four *Fah*$^{-/-}$*Rosa26*$^{+/-}$ recipients. After more than 80% of the liver was repopulated, as assessed by Fah immunohistochemistry, 1×10^5 hepatocytes were serially transplanted into each of two *Fah*$^{-/-}$ recipients and the liver was again repopulated to a donor contribution of more than 80%. We considered that in the event of cell fusion between wild-type donor and *Fah*$^{-/-}$*Rosa26*$^{+/-}$ host hepatocytes in the primary recipients, cells positive for both Fah and β-galactosidase would be selected in the secondary recipients. To determine whether fusion products were present in the secondary recipients, we plated 1×10^7 hepatocytes from each of four mice and stained them for β-galactosidase. We observed no blue hepatocytes among the roughly 3×10^7 Fah-positive cells scored, indicating that the frequency of potential hepatocyte-hepatocyte fusion was less than 1 in 3×10^7 cells. Therefore, secondary hepatocyte fusion is negligible in the *Fah*$^{-/-}$ model and cannot account for the fusion phenotype of BMHs.

Hepatocytes generated by *in vivo* fusion with hematopoietic cells are fully functional and as such have therapeutic promise[1]. To identify the hematopoietic cell with the most potential for therapeutic use, we tested whether the HSC itself or one of its differentiated progeny fuses with host hepatocytes. To determine whether BMHs are derived from cells of the lymphoid lineage, we transplanted HSCs from female *Rag1*$^{-/-}$ mice into congenic male *Fah*$^{-/-}$ recipients. *Rag1*$^{-/-}$ mice show a block in lymphocyte differentiation at the pro-B and pro-T stage and therefore lack circulating mature B and T cells[14]. To avoid the possibility of contamination with nonhematopoietic progenitors, donor c-kit$^+$Sca-1$^+$Lin$^-$ (KSL) cells were double-sorted to homogeneity[15] (Fig. 1b). After hematopoietic engraftment, treatment with the liver protective drug 2-(2-nitro-4-trifluoro-methylbenzol)-1,3-cyclohexanedione (NTBC) was halted to select for Fah-expressing cells[2,13]. BMHs were observed in all transplanted mice after only 6 weeks of liver damage (Fig. 1c and Table 1). As expected, these cells were positive for the Y chromosome (Fig. 1d), indicating that they originated from cell fusion. The frequency of fusion events (Table 1) was similar to that seen after the transplantation of 2×10^6 unfractionated wild-type bone marrow cells into *Fah*$^{-/-}$ recipients[16] and to results obtained in wild-type mice[10]. Thus, B and T cells are dispensable for the generation of BMHs.

Natural killer cells and lymphoid progenitors preceding the pro-B and pro-T stage are, however, still present in *Rag1*$^{-/-}$ mice. To define further the phenotype of the hepatocyte's fusion partner, we capitalized on the identification and phenotypic characterization of GMPs[17]. These lineage-committed progenitors lack self-renewal

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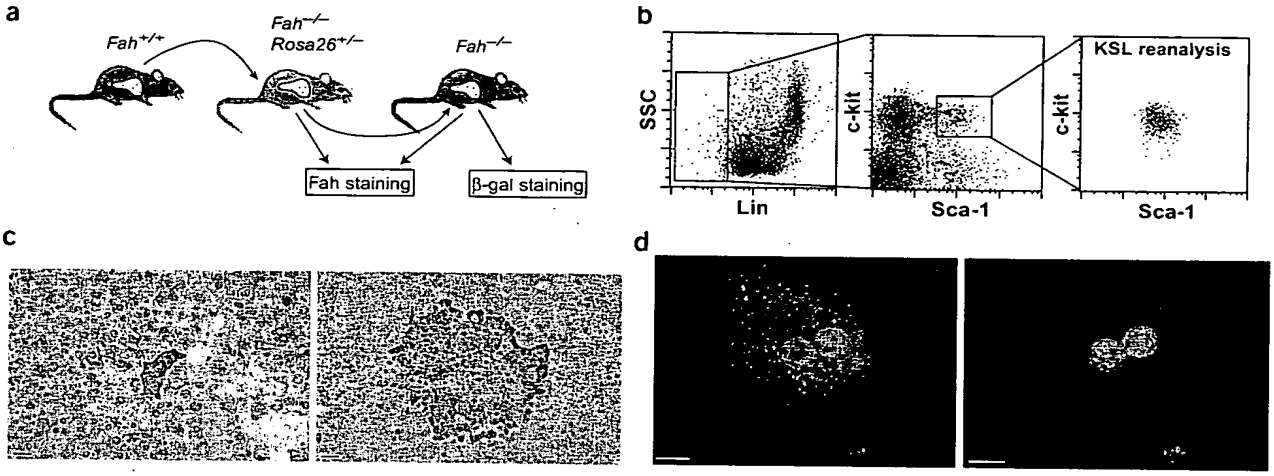


Figure 1 BMHs originate directly from fusion and independently of lymphocytes. (a) Serial hepatocyte transplantation scheme for detecting hepatocyte fusion. (b) Sorting of HSCs from *Rag1*$^{-/-}$ mice. Lineage-negative bone marrow cells (left) were sorted[15] for the coexpression of c-kit and Sca-1 (middle). Double-sorted KSL cells were reanalyzed for purity (right). (c) Fah (brown) immunohistochemistry of a single cell (scale bar, 50 μm) and small nodule (scale bar, 100 μm) of BMHs 17 weeks (6 weeks off NTBC) after the transplantation of *Rag1*$^{-/-}$ HSCs into a *Fah*$^{-/-}$ mouse. (d) Sequential Fah (red) immunocytochemistry (left) and interphase FISH (right; X chromosome, green; Y chromosome, pink) on the same BMH derived from the transplantation of female *Rag1*$^{-/-}$ HSCs into a male *Fah*$^{-/-}$ mouse (scale bars, 20 μm). The characteristic 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining (blue) of both the Fah-positive BMH and the Fah-negative control cell (bottom right) is preserved throughout the procedure. Although interphase FISH with chromosome paints is not suited for exact chromosome counts, it is apparent that both the BMH and the control cell are Y-chromosome positive.

capacity but give rise to a short-term burst of differentiated myelomonocytic cells[18,19]. Although GMPs show no lymphoid activity[17], we used only double-sorted GMP (IL-7Rα$^-$Lin$^-$Sca-1$^-$ c-kit$^+$CD34$^+$FcγRII/III^{hi}) populations to avoid contamination with HSCs or lymphoid progenitors (Fig. 2a). An aliquot of these cells was subjected to activity and purity control in methylcellulose myeloid and B cell progenitor assays, respectively[17,20]. As reported, GMPs gave rise only to colony-forming units (CFU)-granulocyte (CFU-G), CFU-macrophage (CFU-M), CFU-granulocyte/macrophage (CFU-GM) but not burst-forming units-erythroid (BFU-E), CFU-megakaryocyte (CFU-Meg) or B cell colonies, such as are seen in the common myeloid progenitor (CMP)[17] (data not shown). *In vivo*, the myeloid progeny of GMPs were detectable for up to 2 weeks after transplantation, consistent with the transient repopulating capability of these committed progenitors[18,21] (Supplementary Fig. 1 and Supplementary Methods online).

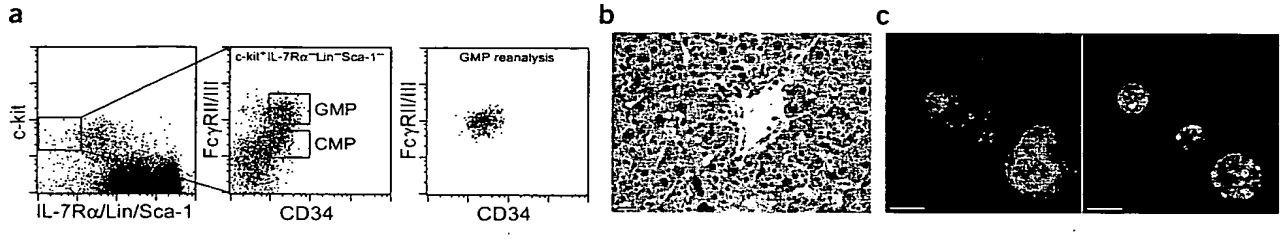
Table 1 BMHs from transplantation of *Rag1*$^{-/-}$ HSCs, GMPs and BMMs

Transplant and recipient	Percentage engraftment		Time of analysis		Clone size[a]	Fusion frequency[b]
	BM	Spleen	Weeks after T	Weeks off NTBC		
HSCs (3×10^3)						
1	60	ND	17	6	1–150 (21)	1/84,000 (1/18,700)
2	50	ND	17	6	1–150 (18)	1/108,000 (1/25,600)
3	50	ND	15	6	1–80 (20)	1/113,000 (1/25,500)
4	50	ND	15	6	1–50 (11)	1/118,500 (1/35,500)
5[c]	ND	ND	27	17	(>250)	4%[d]
GMPs (9×10^3)						
6	0	0	12	6	10–15 (12)	1/395,000 (1/113,800)
7	0	0	12	6	8–20 (13)	1/357,000 (1/99,250)
8	0	0	9	6	1–17 (10)	1/236,500 (1/73,900)
9[c]	ND	0	20	13	(>150)	2%[d]
BMMs (1×10^6)						
10	0	0	19	9	18–30 (25)	1/570,000 (1/114,100)
11	0	0	19	9	1–150 (25)	1/1,107,000 (1/221,500)
12	0	0	9	6	1–15 (8)	1/503,000 (1/176,500)

Data are from 12 mice with BMHs. Bone marrow (BM) and/or splenic engraftment were analyzed by PCR for *Rosa26*, *Fah* or both. In addition, flow cytometry for Ly5.2$^+$ cells in bone marrow and spleen was done for GMP recipients. Liver samples were analyzed by Fah immunohistochemistry at the times indicated (T, transplantation). NTBC withdrawal was done in periods of 3 weeks. ND, not determined.

[a]Range (mean) of Fah-positive cells in a two-dimensional section of a BMH clone. [b]Number of Fah-positive clones per total number of hepatocytes scored, corrected for the increased likelihood of detecting a multicellular clone as compared with a single cell (mean clone sizes were used to determine correction factors)[16]. Uncorrected frequencies are given in parentheses. For each mouse, more than 5×10^5 hepatocytes were scored. [c]Serum bilirubin and ALT of mice 5 and 9 were measured at the times indicated (Fig. 2d). Hematopoietic engraftment of mouse 9 was analyzed by flow cytometry of peripheral blood for Ly5.2$^+$ cells. [d]The percentage replacement of host hepatocytes by BMH is shown.

To test the GMP progeny for hepatocyte fusion potential, male *Fah*$^{-/-}$ mice (Ly5.1) were transplanted with 9×10^3 GMPs derived from congenic female *Fah*$^{+/+}$ donors (Ly5.2). The recipients were conditioned by sublethal irradiation (8 Gy) and NTBC was discontinued immediately after transplantation. We observed single cells or small clusters of Fah-positive BMHs 12 weeks after GMP transplantation (Fig. 2b and Table 1). These BMHs contained Y chromosomes, indicating that they originated by fusion (Fig. 2c). Flow cytometry analysis of the recipients' blood did not detect any donor-derived lymphoid cells (Ly5.2$^+$B220$^+$ or Ly5.2$^+$CD3$^+$; data not shown). In addition, neither bone marrow nor splenic engraftment of donor cells was



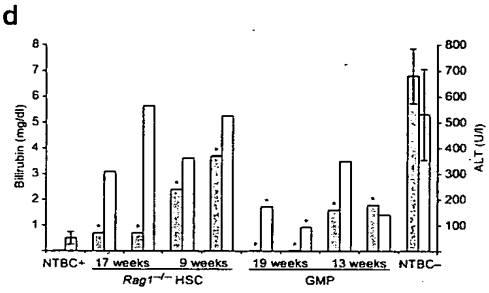
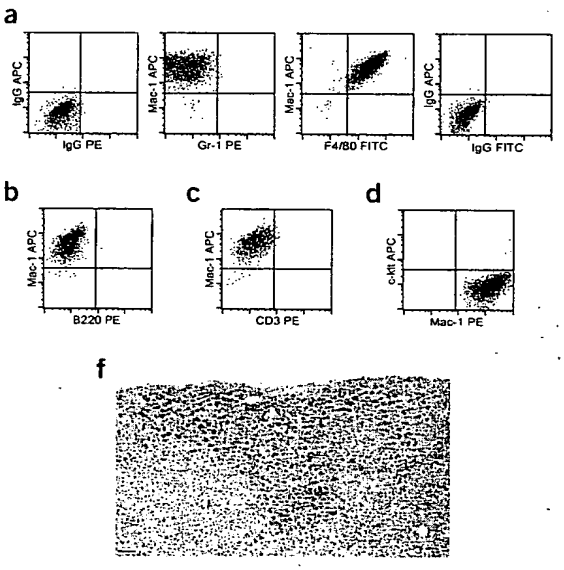


Figure 2 BMHs from transplantation of myelomonocytic progenitors. (a) Left and middle, c-kit-positive cells depleted of IL-7Rα, Sca-1 and lineage marker expression were sorted for GMP and CMP populations as described[17]. Right, reanalysis of double-sorted GMPs. See **Supplementary Figure 2** online for isotype controls. (b) Fah (brown) immunohistochemistry of perivenous BMHs 12 weeks (6 weeks off NTBC) after the transplantation of GMPs into a $Fah^{-/-}$ mouse (scale bar, 50 μm). (c) Sequential Fah (red) immunocytochemistry (left) and interphase FISH (right; colored as in **Fig. 1d**) on the same BMH derived from transplantation of female GMPs into a male $Fah^{-/-}$ mouse (scale bars, 20 μm). Fah-expressing BMHs flanking a Fah-negative cell show Y chromosome signals. (d) Correction of liver function by BMHs. Shown are serum bilirubin (mg/dl, gray bars) and ALT (U/l, white bars) levels in $Fah^{-/-}$ mice after transplantation with $Rag1^{-/-}$ HSCs or GMPs. Controls show the mean ± s.d. for $Fah^{-/-}$ mice on NTBC (NTBC+, n = 14) and 4 weeks off NTBC (NTBC−, n = 8). All mice were analyzed after 4 weeks off NTBC, except for those transplanted with $Rag1^{-/-}$ HSCs, which were off NTBC for 17 weeks and analyzed after 8 consecutive weeks off NTBC. $^*P < 0.03$ versus NTBC− control mice (two-tailed t-test).

detectable by flow cytometry (Ly5.2$^+$) or PCR (*Fah*) analysis (Table 1). Therefore, the transplanted GMP population was devoid of HSCs or lymphoid progenitors[18,20,21].

To assess the functional properties of BMHs derived from fusion with myelomonocytic cells, we analyzed serum bilirubin and alanine aminotransferase (ALT) in mice that had been off NTBC for up to 19 cumulative weeks. Total bilirubin correlates with the functional capacity of the whole organ, whereas ALT levels correspond to the extent of parenchymal cell injury. As a consequence of progressive liver repopulation with Fah-expressing BMHs, both the recipients of $Rag1^{-/-}$ HSCs and those of GMPs showed disease correction with normalization of bilirubin (Fig. 2d). Despite ongoing injury of $Fah^{-/-}$ host hepatocytes, as evident by a lagged decline in ALT, mice that had been off NTBC for 19 or 17 weeks did not require readministration of NTBC. These results suggest that, similar to BMHs derived from wild-type unfractionated bone marrow or HSCs[1], hepatocytes derived by fusion with myelomonocytic cells have normal hepatocellular function and proliferative capability.

The progeny of GMPs include dendritic cells, granulocytes and macrophages, as well as their immediate precursors[17,18]. To determine



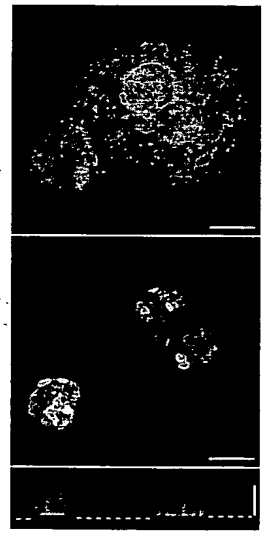


Figure 3 BMHs from transplantation of macrophages. (a–d) Immunophenotyping of BMMs. (a) Macrophage populations generated for transplantation into $Fah^{-/-}$ mice had a pure monocytic phenotype (Mac-1^{hi}Gr-1$^-$F4/80$^+$). Isotype controls for APC/PE and APC/FITC are shown. Contamination with B (b) and T (c) lymphocytes or c-kit+ progenitor cells (d) was excluded. (e) Sequential Fah (red) immunocytochemistry (top) and interphase FISH (middle; colored as in **Fig. 1d**) on the same BMH derived from transplantation of female BMMs into a male $Fah^{-/-}$ mouse (scale bars, 20 μm). The BMH is overlapping a Fah-negative cell that shows fragmentation and dissociation of the nucleus after the FISH procedure. Both cells contain Y chromosomes. A horizontal view generated by deconvolution microscopy (bottom) shows the absence of additional nuclei underneath the DAPI signals (blue) of the Fah-positive and Fah-negative cell (scale bar, 20 μm). The cell culture slide is represented by a broken line. (f) Fah (brown) immunohistochemistry of large nodule of BMHs from transplantation of BMMs after 9 weeks of NTBC withdrawal (scale bar, 200 μm).

definitively whether a mature macrophage population could act as hepatocyte fusion partner, we generated macrophages from the mononuclear fraction of bone marrow from female *Rosa26*$^{+/-}$ mice[22]. Differentiation was induced in a 10-d culture containing mouse macrophage colony-stimulating factor (MCSF) and high serum[23,24]. The resulting BMM population was determined to be essentially pure for the surface marker phenotype Mac-1^{hi}Gr-1^{-}F4/80^{+} (Fig. 3a), which can be found on circulating monocytes and resident macrophages such as Kupffer cells in the liver[25]. Mac-1^{lo-hi}Gr-1^{hi} granulocytes (Fig. 3a), B220^{+} B (Fig. 3b) and CD3^{+} T (Fig. 3c) lymphocytes, as well as c-kit^{+} progenitors (Fig. 3d), were absent from these cultures.

We did not carry out myeloablative conditioning with irradiation, but we used intravenous clodronate liposomes to deplete circulating and resident host macrophages before transplantation[26]. After the injection of 1×10^6 BMMs into the spleens of male *Fah*$^{-/-}$ mice and 6–9 weeks of selection by NTBC withdrawal, we found clusters of up to 150 Fah-positive fusion-derived hepatocytes in three independent recipients (Fig. 3e,f and Table 1). Although engraftment by contaminating stem or progenitor cells would not be anticipated in nonmyeloablated mice and owing to the purity of the transplanted macrophage populations, this possibility was excluded by the absence of donor markers (*Fah* and *Rosa26*), as determined by PCR on DNA derived from the bone marrow and spleen of all recipient mice (data not shown). Thus, differentiated macrophages that do not depend on hematopoietic engraftment are sufficient for generating BMHs.

To show that the ability to form BMHs is not a property of any cell transplanted into the liver, we used CD45^{-}Mac-1^{-} bone marrow stromal cells derived from *Rosa26*$^{+/-}$ mice as donor cells in control experiments. A total of 20 *Fah*$^{-/-}$ mice were each transplanted with 1×10^6 of these cells, with or without sublethal irradiation, but no Fah-expressing hepatocytes were detected in 1×10^7 cells scored 2–7 months after transplantation, despite extensive selection with up to 15 cumulative weeks of NTBC withdrawal (data not shown).

We have shown that the emergence of functional hepatocytes from bone marrow is not dependent on the presence of HSCs. Secondary cell fusion between hepatocytes themselves can also be ruled out as an explanation for the fusion phenotype of BMHs. Both of these findings strongly contradict the hypothesis that stem cell plasticity underlies the ability of HSCs to give rise to cells from different germ-layer lineages. Rather, our results show that cells committed to the myelomonocytic lineage are the dominant source of bone marrow–derived liver epithelial cells, as has been previously suggested for bone marrow–derived myofibers[27].

Fah$^{-/-}$ mice have been shown to carry some aneuploid BMHs after bone marrow transplantation and NTBC withdrawal; however, it is currently unknown whether this is due to unequal chromosome segregation in hybrids or to genomic instability caused by Fah deficiency itself[2,28]. Interphase fluorescence *in situ* hybridization (FISH) with X and Y chromosome paints is not reliably quantitative and, thus, although we did not observe malignant hepatic transformation in any of the mice in this study, the potential hazard posed by aneuploidy demands further investigation.

Activation of Fah in BMHs reflects reprogramming of the hematopoietic nucleus to a hepatocyte expression pattern, and our results show that differentiated macrophages are suitable candidates for reprogramming strategies. Further insight into the nature of reprogramming and other factors that govern *in vivo* cell fusion should improve its efficiency to levels that are therapeutically relevant without requiring the positive selection inherent to Fah deficiency. Because HSCs and stable hematopoietic engraftment are not required to make 'liver from blood', the administration of fusogenic macrophages to the damaged organ either directly or by systemic transplantation of their highly proliferative progenitors would represent a more targeted and less invasive therapeutic strategy than bone marrow transplantation.

METHODS

Mice. For hematopoietic cell transplantations, female C57BL/6 *Rag1*$^{-/-}$ (ref. 14), C57BL/6 Ka-Thy1.1-Ly5.2 (ref. 29) and C57BL/6 *Rosa26* (ref. 22) donor mice and male C57BL/6 *Fah*$^{-/-}$ (ref. 30) recipient mice were used. All mouse experiments were approved by the Institutional Animal Care and Use Committee of the Oregon Health & Science University.

Cell staining and sorting. For KSL cells, single-cell suspensions of bone marrow were labeled with the allophycocyanin (APC)-conjugated antibodies to c-kit, fluorescein isothiocyanate (FITC)-conjugated antibodies to Ly6AE (Sca-1) and a phycoerythrin (PE)-conjugated lineage mixture (B220, CD3, CD5, CD4, CD8, Mac-1, Gr-1 and Ter119). KSL cells were enriched to homogeneity by double-sorting of c-kit^{+}Sca-1^{+}Lin^{-} cells. For myeloid progenitors, we labeled cells with a PE-conjugated lineage mixture (B220, CD3, CD4, CD8, Gr-1, CD19, IgM (e-Biosciences) and Ter119) and anti-IL-7Rα–PE (e-Biosciences), anti-Ly6AE–PE, anti-c-kit–PharRed (APC-Cy7; e-Biosciences), anti-CD34–FITC and anti-FcγRII/III–APC (e-Biosciences) antibodies. Purified progenitors were obtained by double sorting of IL-7Rα^{-}Lin^{-}Sca-1^{-}c-kit^{+}CD34^{+}FcγRII/III^{lo} (CMP) and IL-7Rα^{-}Lin^{-}Sca-1^{-}c-kit^{+}CD34^{+}FcγRII/III^{hi} (GMP) populations.

Hematopoietic engraftment was analyzed with anti-Ly5.2–FITC, anti-Mac-1–APC, anti-B220–APC and anti-CD3–APC antibodies. Peripheral blood leukocytes were obtained by retro-orbital bleeding, followed by erythrocyte depletion by sedimentation in 3% dextran (Amersham Pharmacia) and hypotonic lysis. Immunophenotyping of BMMs was done with anti-Mac-1–APC, anti-Gr-1–PE, anti-F4/80–FITC (Serotec), anti-B220–PE, anti-CD3–PE and anti-c-kit–APC antibodies. We excluded dead cells by using a combination of scatter gates and propidium iodide staining. All antibodies were purchased from PharMingen unless indicated otherwise. Sorting procedures and analyses were done on a FACS Vantage (Becton Dickinson).

GMP and CMP colony assays. Double-sorted progenitors were cultured in Iscove's modified Dulbecco's medium (IMDM)-based methylcellulose media (Methocult GF M3434; StemCell Technologies) supplemented with 50 ng/ml of mouse stem cell factor (SCF), 10 ng/ml of mouse IL-3, 10 ng/ml of human IL-6 and 3 U/ml of human erythropoietin by the manufacturer to support the growth of CFU-G, CFU-M, CFU-GM and BFU-E. To promote additional growth of CFU-Meg, we added 10 ng/ml of mouse thrombopoietin (StemCell Technologies) at the start of the culture. Cultures contained 200 progenitor cells per milliliter and were scored at day 7. To detect lymphoid colony-forming activity, we used IMDM-based Methocult M3630 (StemCell Technologies) containing 10 ng/ml of human IL-7 and supplemented with 50 ng/ml of mouse SCF (Stem Cell Technologies). These pre-B cell colony assays contained 1,000 progenitor cells per milliliter and were observed up until day 14. All assays were plated in 35-mm dishes (Corning) and incubated at 37 °C in a humidified chamber under 5% CO_2.

Macrophage generation. The mononuclear fraction of bone marrow was isolated by gradient centrifugation (500*g* for 20 min) using NycoPrep 1.077A (Axis-Shield). Cells were plated at a density of 2.5×10^6/ml in Dulbecco's modified Eagle's medium (DMEM; Gibco/Invitrogen) supplemented with 15% fetal calf serum (FCS; Hyclone) and 20 ng/ml of mouse MCSF (R&D Systems) in 75-cm^2 flasks (Corning). Nonadherent cells were separated and replated after 24 h and incubated for 9 d with medium exchange every 3 d. For transplantation, cells were detached with 1.0 mg/ml of Dispase II (Roche Applied Science).

Transplantations. Single-hepatocyte suspensions were generated by collagenase perfusion and were injected into the spleen[13]. Myeloablative conditioning was done by two applications (3 h apart) of 6 Gy of total body irradiation using a ^{137}Cs γ-source. For sublethal irradiation, 8 Gy was applied. We injected KSL cells and GMPs into the retro-orbital venous plexus. BMMs were injected into the spleen 1 d after intravenous administration of clodronate liposomes[26]. NTBC withdrawal was done in periods of 3 weeks. The first three cycles off

NTBC were separated by recovery phases of 3 weeks on NTBC. For further cycles off NTBC, recovery phases of 1–2 weeks were sufficient. After HSC transplantation, an initial hematopoietic engraftment period of 3 weeks on NTBC was allowed.

Fah and β-galactosidase analyses. PCR analyses for *Fah* and *Rosa26* were done as described[2,13]. Immunohistochemistry for Fah was done as described[1,2]. To measure the frequency of BMHs in sections, at least 5×10^5 hepatocytes were scored[16]. We generated single-hepatocyte suspensions as described[13] and cultured them in low-glucose DMEM medium supplemented with 10% FCS, 100 ng/ml of human epidermal growth factor (Gibco/Invitrogen) and 1× Insulin/Transferrin/Selenium-A (Gibco/Invitrogen). Detection of β-galactosidase expression was done by cytochemically staining hepatocytes plated on Primaria dishes (Falcon/Becton Dickinson Labware)[2]. The sensitivity of this assay was determined to be less than 1 in 1×10^6 cells on the basis of mixing experiments with *Rosa26*$^{+/-}$ and *Rosa26*$^{-/-}$ hepatocytes.

For Fah immunocytochemistry, hepatocytes were plated on LabTek II CC2 (Nalge Nunc) chamber slides at a density of 1×10^4 cells per cm^2. Four hours after plating, cells were fixed in 4% paraformaldehyde (Sigma) and permeabilized with 0.25% Triton (Sigma). Blocking was done in 10% FCS. For Fah detection, cells were first incubated with polyclonal rabbit antibody to Fah diluted 1:10,000 and then incubated with alkaline phosphatase–conjugated swine anti-rabbit antibody (DakoCytomation) diluted 1:25. The Fast Red Substrate System (DakoCytomation) was used for visualization.

Cytogenetics. For sequential Fah staining and sex chromosome FISH, bright-field and fluorescent images of cleanly isolated Fah-positive cells were captured by using a Nikon E8000 fluorescent microscope and CytoVision software (Applied Imaging). Subsequent interphase FISH with paints staining the whole mouse X and Y chromosome (Vysis) was done as described[2]. We used three-dimensional imaging by deconvolution microscopy with the DeltaVision system (Applied Precision) to rule out the presence of additional cells underneath the BMHs analyzed for this study.

Note: Supplementary information is available on the Nature Medicine website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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Features of skin-coincubated macrophages that promote recovery from spinal cord injury

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Abstract

Uncontrolled inflammation is considered to exacerbate the neuronal loss that follows spinal cord trauma. However, controlled inflammation response appears to be beneficial. Skin-coincubated macrophages injected into contused spinal cord of rats resulted in improved motor recovery and reduced spinal cyst formation. The macrophages express elevated levels of cell-surface molecules CD80, CD86, CD54 and MHC-II, markers characteristic of antigen presenting cells (APCs). Additionally, skin-coincubation elevates secretion of interleukin-1β (IL-1β) and Brain-Derived Neurotrophic Factor (BDNF), and reduces secretion of tumor necrosis factor α (TNF-α). We propose that macrophages activated by skin-coincubation bolster neuroprotective immune activity in the spinal cord, making the environment less cytotoxic and less hostile to axonal regeneration.

Keywords: Central nervous system; Proinflammatory cytokines; Macrophage activation; Spinal cord injury; Syringomyelia

1. Introduction

Severe spinal cord injury results in an irreversible loss of motor and sensory functions below the level of injury due to the hostility of the environment to fiber regeneration, as well as the extremely poor wound-healing capacity of the mature central nervous system (CNS). In addition to the mechanical, chemical or metabolic injury sustained as a result of the primary insult, the damaged tissue secretes substances that become toxic, damaging still-viable cells in the vicinity. Such secondary degeneration leads to further functional loss.

The immune system plays a key role in the healing of injured tissues (Sicard, 2002), with the immune cells scavenging toxic elements and secreting trophic factors (Wilson, 1997). These healing processes allow regeneration of damaged peripheral nerves (Muller and Stoll, 1998; Kury et al., 2001), but are much less active in repairing CNS damage. Following injury to the spinal cord, the acute cellular inflammatory response involves activation of resident microglia and infiltration of blood-borne leukocytes (Carlson et al., 1998; Schnell et al., 1999; Butovsky et al., 2001). Among the factors that determine the effect of such a response are its onset, shut off, its phenotype and intensity. These factors determine whether the immune response is detrimental, insufficient or beneficial for nerve healing (Bethea and Dietrich, 2002; Hauben and Schwartz, 2003).

Over the years, it became clear that there are several local and systemic methods to boost and/or modulate the immune response, leading to CNS repair (Rapalino et al., 1998; Bethea et al., 1999; Hauben et al., 2000, 2001a,b; Bethea and Dietrich, 2002; Ellezam et al., 2003; Hauben and Schwartz, 2003; Hofstetter et al., 2003). One such modulation method involves the local injection of autologous macrophages that had been activated to a wound-healing phenotype through coincubation with peripheral nerve segments (Lazarov-Spiegler et al., 1998). This led to significant motor recovery in rats with transected spinal cords (Rapalino et al., 1998).

In developing a feasible autologous macrophage therapy for spinal cord injury, we have adopted skin as the source of activating tissue. We have characterized the phenotype of these macrophages, and tested their potency in a second

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model that involves severe spinal cord contusion (Basso et al., 1996; Young, 2002). Like the transection model, the contusion model leads to severe paralysis with very poor motor recovery in the absence of intervention. The spinal cord contusion animal model is considered to be representative of the human pathology of spinal cord injury (Schwab, 2002). In the present study, the macrophage therapy was prepared from spinally contused donor rats and administered to the recipients 8–9 days after injury, to mimic the typical clinical use of macrophages as an autologous cell therapy for spinal cord injury. We demonstrate that blood-borne monocytes activated ex vivo by skin acquired a characteristic secretory profile, and a phenotype reminiscent of antigen presenting cells (APCs). These cells were shown to contribute to functional and morphological recovery following contusion.

2. Materials and methods

2.1. Animals

Adult (8–10 weeks) male Sprague–Dawley (SPD) rats (210–230 g) supplied by Harlan laboratories (Israel) were used in all the experiments. Animal care was performed according to the regulations formulated by Institutional Animal Care and Use Committee (IACUC). Rats that had undergone spinal cord contusion (see below) were treated with prophylactic antibiotics (Trimethoprim 1.6 mg/rat, Sulphamethoxazole 8 mg/rat twice a day, Teva Pharmaceuticals, Petach-Tikva, Israel) and underwent urinary bladder massage at least twice a day until recovery of spontaneous micturition.

2.2. Spinal cord contusion

Rats were anesthetized (Ketamine 70 mg/kg, Bedford Laboratories, OH, US, Xylazine 10 mg/kg, VMP, Bioulab, France), laminectomized at T9, and contused using a NYU impactor (Gruner, 1992) to drop a 10-g metal rod from height of 50 mm onto the exposed spinal cord (considered to cause severe damage).

2.3. Purification of rat monocytes

Rat monocytes were purified from whole blood of SPD male rats. The rats were anesthetized by intraperitoneal injection of Pentobarbitone (300 mg/kg, CTS, Chemical Industries, Israel) and blood was drained from the heart. The heparinized blood was diluted 1:4 with PBS, overlaid on Percoll (final density = 1.0825 g/ml, Amersham Biosciences, Uppsala, Sweden) and subjected to centrifugation at $490 \times g$ at room temperature for 32 min. The mononuclear cells in the upper band ($\sim 10 \times 10^6$ cells/ml) were collected and lymphocytes were removed using streptavidin-conjugated beads (GenoVision, Exton, PA) preloaded with biotinylated antibodies specific for T cells and B cells (anti-CD3 and anti-CD45RA, Serotec, Kidlington, UK). The following quantities were used for every 10^6 mononuclear cells: for T-cells, 17 μg beads and 0.3 μg anti-CD3 antibodies; for B-cells, 5 μg beads and 0.1 μg anti-CD45RA antibodies. The cell fraction was incubated with the beads for 30 min at 4 °C, then loaded onto Percoll and centrifuged at $490 \times g$ for 32 min at 10 °C. Finally, the purified monocyte fraction was collected from the upper band.

2.4. Skin preparation and coincubation with monocytes

Small pieces of skin (2 × 6 mm) were prepared from the backs of the same donor rats that had been bled to prepare the monocytes. The fur on the upper back was shaved and the skin was sterilized with ethanol before cutting. Two pieces of the skin tissue were placed with 5×10^6 cells of the homologous monocyte fraction (see above) in 5 ml DCCM-1 (Biological Industries, Beit HaEmek, Israel) and incubated for 16 h at 37 °C, 5% CO_2. At the end of incubation, the skin pieces were removed and the cells recovered by centrifugation.

2.5. Analysis of cell phenotypes

The analysis of cell-surface antigens CD86, CD54, MHC class II and CD80 on the monocytes was performed by labeling with phycoerythrin-conjugated antibodies using a flow cytometer (FACSCalibur, Becton Dickinson, San Jose, CA, USA). Mouse anti-CD86 (clone 24F) was purchased from Pharmingen (San Diego, CA, USA). Mouse anti-CD54 (clone 1A29), mouse anti-MHC class II (clone OX-6) and mouse anti-CD80 (clone 3H5) were purchased from Serotec. The cells (0.3×10^6) were initially incubated with PE-labeled antibodies directed against one of the target surface antigens. Subsequently, the cells were fixed and permeabilized (Leucoperm kit, Serotec), and finally, labeled by intracellular staining with fluorescein isothiocyanate-conjugated (FITC) antibodies specific for ED1 (Serotec). Flow cytometry was carried out to evaluate the presence and intensity of each target molecule on ED1-positive cells.

2.6. Secretion of factors

Before and after coincubation with skin, monocytes were assayed for their potential to secrete various soluble factors. The cells were resuspended in fresh DCCM-1 medium (2×10^6/ml) and incubated for 6 h at 37 °C, 5% CO_2. Prior to assay, the supernatants were collected and frozen at − 70 °C. Cytokine levels were quantified by immunoassay using commercial ELISA kits according to the manufacturers' instructions. Tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) were measured using kits from Biosource (Camarillo, CA, USA). For Brain-Derived Neurotrophic Factor (BDNF) measurement, the ELISA kit produced by Chemicon (Temecula, CA, USA) was used.

2.7. Implantation of macrophages

Four to nine days after spinal cord contusion, the incubated rat macrophages—$0.2-0.25 \times 10^6$ ED1-positive cells resuspended in 5–7 μl phosphate-buffered saline (PBS)—were administered by injection into the caudal border of the lesion. Hamilton syringes fitted with 30 G needles were used. Control spinally contused rats were injected in the same manner with medium or left non-injected.

2.8. Behavioral and locomotion follow-up

Macrophage-treated and control animals were examined for functional recovery (defined as voluntary motor function) by evaluating locomotor performance in an open field score (Basso et al., 1995) that ranges from 0 (complete hind-limb paralysis) to 21 (normal movement). All results were assessed according to the observation of two observers who were blinded to the treatment that the animals had received. Starting at 1 week after the contusion, rat behavior was assessed weekly. The follow-up was continued for at least 2 months until a steady state was reached. Rats were considered to have achieved meaningful recovery if the locomotor score was at least 6 in one hind limb (extensive movement of two joints and partial movement of the third joint) at two or more evaluation sessions.

2.9. Morphology

Five to six months after contusion, macrophage-treated and control rats were sacrificed and perfused with PBS containing 4% paraformaldehyde. The fixed spinal cords

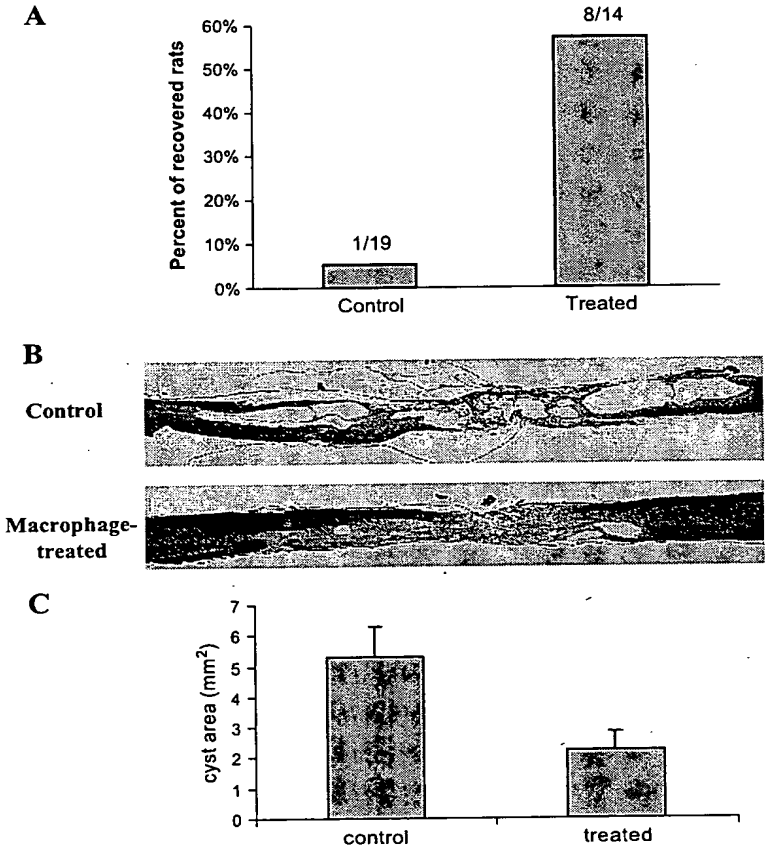

Fig. 1. In vivo effect of macrophage treatment on spinal cord of contused rats. (A) Effect of macrophage treatment on recovery of motor activity after spinal contusion. The skin-coincubated cells (200,000–250,000 of ED1 positive cells) were implanted 8–9 days after the contusion. Numbers of recovered rats (with locomotor score ≥ 6), indicated as fractions above the bars, differed significantly between macrophage-treated and control animals ($p = 0.0015$, Fisher exact test, one-tail). (B) Representative longitudinal sections of spinal cord from a rat treated with rat macrophages and a control rat (injected with vehicle) 5 to 6 months after spinal contusion. The sections shown are 20 mm long and stained with Sudan black. (C) Cavity areas in longitudinal sections of rat spinal cords 5 to 6 months after contusion. Fixed spinal cords from the rats ($n = 9$ rats for controls, $n = 5$ rats for macrophage-treated) were sectioned longitudinally and three slices per cord (central, ventral and dorsal) were evaluated for cysts. The control rats include three injected with medium and six non-injected (no significant difference between them). The macrophage-treated group consisted of five rats injected with macrophages 4 days after contusion. The results, presented as mean ± S.E., show a significant treatment-related difference (t-test with unequal variances, $p = 0.024$).

were removed and frozen using sucrose as the cryoprotectant. The cords were then sectioned longitudinally using a cryostat (20 μm/section). Following staining with 0.3% Sudan Black B (SBB, Merck, Darmstadt, Germany), three sections per spinal cord (ventral, central and dorsal) were examined under a laser scanning confocal microscope (Zeiss, LSM510) and digital images were recorded. The areas of the cavities in the spinal cord sections were evaluated using ScionImage software (Scion, Maryland, USA).

3. Results

3.1. In vivo effect of skin-coincubated macrophages

The aim of the present study has been to characterize the features of skin-coincubated macrophages promote recovery from spinal cord injury. Our criteria for in vivo recovery are based on functional and morphological assessments.

Functional recovery of spinally contused rats was measured using the open field motor score. Improvement in functional recovery was evident, with significantly more macrophage-treated animals showing meaningful recovery (a motor score of 6 or above) than control animals. Fig. 1A shows the results, obtained from 19 control animals (10 non-injected, 9 injected with medium) and 14 macrophage-treated animals. Skin-coincubated macrophages or vehicle was injected caudally to the lesion site 9 days after the contusion and animals were followed by assessing motor scores for 60 days.

One of the common complications after spinal cord injury is the formation of fluid-filled cavities or cysts (syringomyelia) several months to decades after the injury (Kramer and Levine, 1997). We therefore examined cyst formation in spinal cords of rats treated and non-treated with skin-coincubated monocytes.

Fig. 1B shows representative photographs of longitudinal sections of contused spinal cords of a control and a macrophage-treated animal excised 5 to 6 months after the contusion. As can be seen in the micrographs, both sections show cavities caudally and rostrally to the site of injury, but they are much less extensive in the macrophage-treated spinal cord. Fig. 1C shows the results of the quantitative analysis of areas occupied by the cysts measured in spinal cord sections (three per animal) of five macrophage-treated animals and nine controls. This revealed a large, statistically significant difference (t-test for unequal variances, $p = 0.024$) in cyst

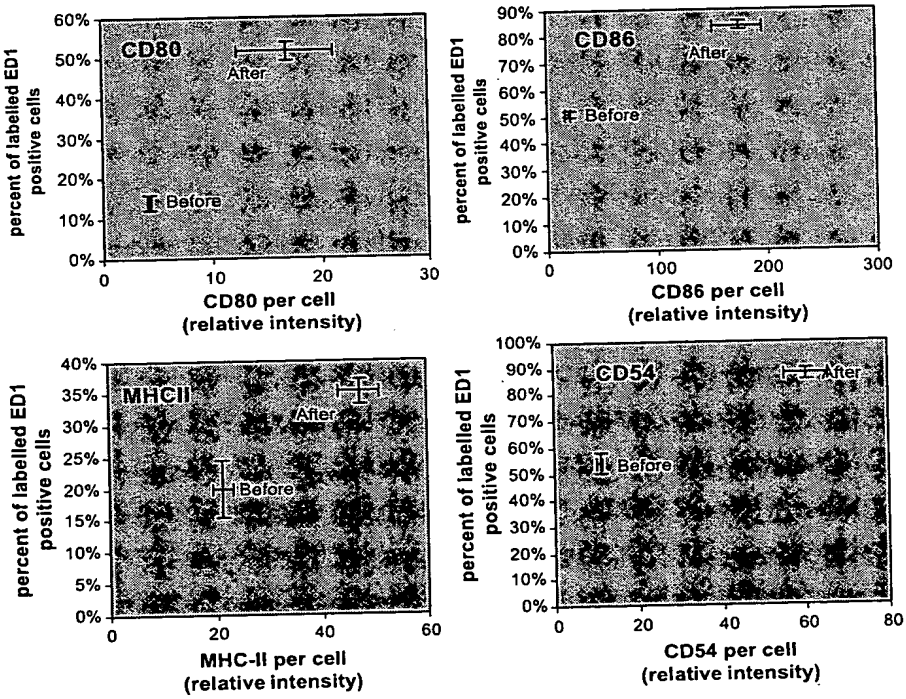

Fig. 2. Expression of membrane markers on ED1 positive cells. The blood-borne monocytes before and after coincubation with skin were double-labeled with antibodies against ED1 together with antibodies against target molecule CD80, CD86, CD54 or MHC class II. The plots represent percent of ED1 positive cells carrying the target molecule (vertical axis) and the mean expression of the target molecule per ED1 positive cell (horizontal axis). Each point represents the average for analysis of 16 or 17 independent batches with its standard errors. The differences in both parameters between cells before and after incubation with skin were significant for each target molecule (paired t-tests, $p < 0.01$ in all cases).

area between macrophage-treated and control rats. A correlation between size of cavity and motor scores in the analyzed animals of both groups combined gave a Spearman correlation coefficient of -0.63 (with significance at $p=0.016$).

3.2. Characterization of macrophages

The expression of molecules characteristic of antigen-presenting cells (APCs) was analyzed. The analysis included costimulatory factors CD80 (B7-1) and CD86 (B7-2), MHC class II molecule OX-6, and adhesion molecule CD54 (ICAM1). In each case, the probe for the target molecule was applied together with a probe for ED1 (a marker for macrophages) in a double-labeling procedure, allowing selective analysis of ED1-positive cells only. Each target molecule was assayed in numerous ($n \geq 16$) cell batches, in terms of the percentage of ED1-positive cells expressing it, and its intensity of expression per ED1-positive cell (Fig. 2). For all four target molecules, both of the measured parameters were significantly larger in the cells after skin-coincubation (paired t-test, $p<0.01$).

Cytokine secretion by the macrophages—specifically TNF-α, IL-1β, and IL-6, pro-inflammatory cytokines typical of macrophage activation—was assayed before and after skin coincubation. Secretions of each cytokine per million ED1-positive cells are presented in Fig. 3. TNF-α showed a significant decrease following skin activation, IL-6 showed no significant change, whereas IL-1β showed a significant increase.

A similar analysis was performed on the secretion of BDNF by macrophages before and after skin coincubation. Fig. 4 shows a significant increase in BDNF secretion per million ED1-positive cells following coincubation.

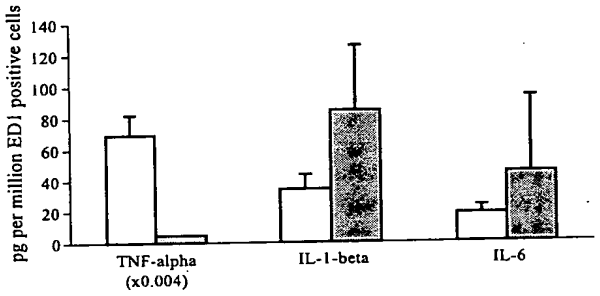

Fig. 3. Secretion of cytokines. The level of secreted cytokines was measured in conditioned media from 2×10^6 cells/ml incubated for 6 h. Cytokine levels were assayed by ELISA and the results were calculated per million ED1-positive cells. The values given represent the averages (columns) and standard errors (error bars) for several batches (TNF-α, 9 batches; IL-1β, 9 batches and IL-6, 8 batches). The TNFα results have been scaled down by a factor of 250 to fit the axis. Paired t-test to compare secretion levels before and after coincubation with skin showed significant differences in levels of TNF-α ($p=0.01$) and IL-1β ($p=0.02$).

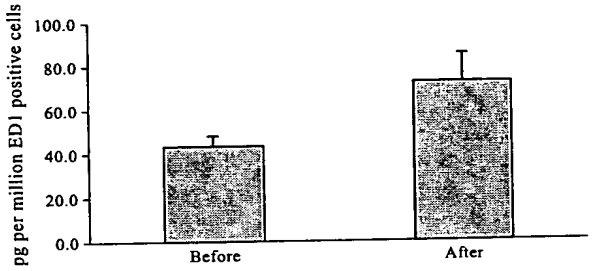

Fig. 4. Secretion of Brain-Derived Neurotrophic Factor (BDNF). The secretion of BDNF was measured in the same manner as the other molecules described in Fig. 3. The results shown were obtained from eight batches. The paired t-test showed significant difference between the levels of BDNF before and after incubation with skin ($p=0.013$).

4. Discussion

We have shown that skin-coincubated macrophages that promote spinal cord recovery express features reminiscent of antigen presenting cells (APCs). Their cytokine secretion profiles are characterized by reduction in TNF-α, elevation of IL-1β and little change in IL-6 in comparison to monocytes before activation. The skin-coincubated macrophages also show enhanced BDNF secretion.

In the past, inflammation has had a bad reputation in the context of central nervous system (CNS) injury in general, and spinal cord injury, in particular. Concepts such as the immune-privileged status of the CNS, as well as observations such as the presence of immune cells in the diseased CNS, fostered the prevailing belief that immune activity in the CNS is detrimental (Lotan and Schwartz, 1994). Many authors consider inflammation to be an important mediator of secondary damage (Dusart and Schwab, 1994; Carlson et al., 1998; Popovich et al., 1999; Mautes et al., 2000; Ghirnikar et al., 2001). Current clinical practice for spinal cord injury is to administer high-dose of methylprednisolone, an anti-inflammatory steroid, within the first hours after injury.

Inflammation is the body's mechanism of tissue repair, promoting clearance of cell debris and secretion of neurotrophic factors and cytokines. It is thus not surprising that a well-controlled immune response, rather than causing damage, was found capable of actually promoting axonal regeneration and spinal cord repair (David et al., 1990; Perry and Brown, 1992; Guth et al., 1994; Huang et al., 1999; Bieber et al., 2001).

These and other results raised the possibility that what determines the ability of inflammation to support healing is the phenotype of the activated macrophages or resident microglia. With these studies, it became clear that describing inflammation as a single phenomenon is too simplistic and ignores the diversity of phenotypes and their varying destructive or beneficial potentials.

In the present study, we found that skin-coincubated macrophages that promote spinal cord recovery express markers characteristics of APCs. We have also examined their cytokine secretion profiles. New data accumulated in the last decade suggest that monocytes are capable of expressing different functional programs in response to distinct micro-environmental signals. The two most characterized phenotypes are the "classically" and "alternatively" activated macrophages. Classical activation typically involves cellular adaptive immunity, and is characterized by the secretion of pro-inflammatory cytokines with the ability to kill target cells. Alternatively activated macrophages, induced by IL-4 or glucocorticoids, modulate inflammatory responses and adaptive immunity, scavenge debris, and promote angiogenesis, tissue remodeling and repair (Mantovani et al., 2002; Gordon, 2003; Mosser, 2003). In addition to these two well-defined phenotypes, macrophages acquire a variety of other phenotypes in response to various inflammatory and immune stimuli, reflected in their cytokine secretion profile and cellular markers, and consequently affecting the way they interact with other cells.

The classical activation of macrophages using lipopolysaccharide or zymosan (a pathogen-derived material) is characterized by a profound increase in production of IL-1β and TNF-α (Young et al., 2001), and local injection of zymosan to healthy spinal cord has been found induce destructive, uncontrolled inflammation by classical activation of the resident microglia (Popovich et al., 2002). In contrast, by promoting neurological recovery, skin-coincubated macrophages have the opposite effect. Accordingly, it is highly misleading to consider that skin-coincubated macrophages and zymosan-activated microglia cause equivalent inflammatory effects. Furthermore, injection of large doses of skin-coincubated macrophages into the undamaged spinal cord of rats caused no apparent anomalies in clinical or histopathological parameters (unpublished data from animal safety studies). It is thus misleading to consider macrophage activation and inflammatory conditions as uniform processes (Schwartz and Hauben, 2002; Hauben and Schwartz, 2003).

Based on the cellular characteristics that have been determined for skin-coincubated macrophages, we suggest a number of possible mechanisms by which they may act after administration into the damaged spinal cord. The acquisition of APC characteristics by the activated macrophages may provide one mechanism of action to explain the restoration of neurological function. This is because APC-competent macrophages introduced into the damaged tissue would be expected to pick up local antigens, such as myelin basic protein (MBP), and activate infiltrating T cells. Recent studies have shown that MBP-preloaded dendritic cells (professional APCs) promote recovery from spinal cord injury (Bomstein et al., 2002). It has also been shown that T cells specific for MBP protected neurological function in animals subjected to spinal cord contusion, improving their recovery (Hauben et al., 2000). This is consistent with the finding that autoimmune helper T cells benefit CNS tissue repair after aseptic injury but pathology develops if pathogen-related antigens are present (Hofstetter et al., 2003). Suitably activated T cells were shown to regulate microglial cells (Butovsky et al., 2001), increasing their ability to clear noxious elements from the lesion site. We therefore propose that the implanted macrophages in the present study enhance the same neuroprotective cascade of events.

Also of possible relevance to the mechanism of action is the ability of the skin-coincubated macrophages to generate BDNF. Other work has provided evidence that immune cells can be a source of neurotrophic factors (Moalem et al., 2000; Barouch et al., 2001). It was shown that following CNS injury, macrophages and activated glial cells produce local trophic gradients of BDNF and other factors that are suggested to stimulate axonal sprouting (Dougherty et al., 2000; Pataky et al., 2000; Batchelor et al., 2002). Moreover, exogenous administration of BDNF was shown to provide neuroprotection, and to enhance regenerative activity after spinal cord injury (Namiki et al., 2000; Jin et al., 2002; Sayer et al., 2002).

Ultimately, the effects of macrophages demonstrated in our study may include a complex interplay of several mechanisms. The pieces of skin tissue we used to activate the blood-borne monocytes are a damaged tissue with capability to regenerate. As such, they activate macrophages towards a wound-healing phenotype. It is therefore expected that such cells would have the capability to confer benefit to the damaged CNS directly, by secreting trophic factors and removing toxic elements, as well as indirectly through their effect on the local immune activity via cytokine signaling and activation of the adaptive immune response.

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